Wnt Signalling in the Regulation of Synapse Formation and Maintenance

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Declaration of authorship

I, Ellen Marie Dickins, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

The formation of functional neural circuits depends upon a huge excess of synapses forming, many of which are subsequently eliminated in an activity-dependent manner. Critically, robust mechanisms must be in place to ensure selected synapses are maintained. The coordinated gain, loss and maintenance of synapses require a complex molecular dialogue between pre- and postsynaptic neurons involving multiple signals. In addition to establishing functional neuronal circuits, normal brain function requires the correct balance of excitatory and inhibitory synapses. The distribution of excitatory and inhibitory inputs on a single neuron impacts significantly on its output, which in turn determines circuit formation and function. Whilst progress has been made in elucidating mechanisms that regulate synapse assembly, disassembly and maintenance, and the ratio between excitatory and inhibitory synapses, many aspects remain poorly understood.

Wnt proteins are a diverse family of secreted glycoproteins known to stimulate synaptogenesis via Gsk3β. The aim of my thesis has been to further characterize the Wnt signaling pathway and determine whether Wnt7a is a pan-synaptogenic factor or if it preferentially stimulates the assembly of excitatory synapses. I have also explored a role for Wnt-mediated synaptic maintenance in mature synapses. My studies reveal that Wnt7a signals through a divergent-canonical Wnt pathway that is independent of transcription to specifically regulate presynaptic differentiation of excitatory synapses; inhibitory synapses are not affected.

I also found that blockade of endogenous Wnt induces a rapid loss of synapses. Synapse disassembly was revealed by a coordinated loss of multiple pre- and postsynaptic proteins and neurotransmitter release sites. Ultrastructural analyses of remaining synapses revealed significant shrinkage of the active zone and postsynaptic density. To further explore synapse disassembly during Wnt-blockade, I imaged mature neurons expressing VAMP2-mRFP using time-lapse microscopy. Here I found that blockade of endogenous Wnt signaling rapidly induces disassembly of stable synaptic vesicle clusters. My results demonstrate that Wnt signaling plays two roles in hippocampal neurons. Firstly, Wnts promote the assembly of nascent excitatory synapses in young cultures. Secondly, Wnt signaling is required for synaptic maintenance in mature neurons. Synaptic maintenance is a novel role for Wnt signaling at synapses and this thesis work provides new insights into the mechanisms by which Wnt signaling modulates synapses in hippocampal neurons.

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List of abbreviations

Aβ	Amyloid-β
AchR	Acetylcholine receptor
AD	Alzheimer's disease
AmD	Actinomycin D
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
APC	Adenomatous polyposis coli
AZ	Active zone
Aβ	Amyloid-β
BDNF	Brain derived neurotrophic factor
CAM	Cell-adhesion molecule
CAZ	Cytomatrix at the active zone
CF	Climbing fiber
CK	Caesin kinase
CK1	Caesin kinase -1
CNS	Central nervous system
CRD	Cysteine rich domain
Dkk	Dickopf
Dvl	Dishevelled
ECM	Extracellular matrix
EE	Environmental enrichment
EOAD	Early onset Alzheimer's disease
EPSC	Excitatory postsynaptic potential
ER	Endoplasmic reticulum
F-actin	Filamentous actin polymers
FAD	Familial Alzheimer's disease
FGFs	Fibroblast growth factors
FRAP	Fluorescent recovery after photobleaching
FRET	Fluorescence resonance energy transfer
Fz	Frizzled
G-actin	Globular actin
GABA	Y-aminobutyric acid
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
GSK3 β	Glycogen synthase kinase-3β
HSPG	Heparin sulphate proteoglycan
IPSC	Inhibitory postsynaptic potential
IVM	Inter-vesicular matrix
LEF	Lymphoid enhancer factor
LGN	Lateral geniculate nucleus

LOAD	Late onset Alzheimer's disease
LRP	Low-density lipoprotein receptor related
LTD	Long-term depression
LTP	Long-term potentiation
Munc	Mouse uncoordinated
NCAM	Neural cell adhesion molecule
NFAT	Nuclear factor of activated T-cells
NL	Neuroligin
NMDA	N-methyl-d-aspartate
NMJ	Neuromuscular junction
NX	Neurexin
NX	Neurexin
ODC	Occular dominance column
PC	Purkinje cell
PCP	Planar cell polarity pathway
PD	Parkinson's disease
PDZ	PSD-95 discs large ZO-1 homologous
PF	Parallel fiber
PIP_2	Phosphatidylinositol-4,5-biphosphate
PRPs	Plasticity related proteins
PSD	Post synaptic density
PVT	Picollo transport vesicle
RGC	Retinogeniculate cell
RRP	Ready releasable pool (of synaptic vesicles)
Sfrp	Secreted frizzled related protein
SNARE	Soluble NSF attachment protein receptors
SPRC	Synapse associated polyribosome complex
SV	Synaptic vesicle
SVT	SV protein transport vesicle
SynCAM	Synaptic cell adhesion molecule
TCF	T-cell factor
TSP	Thrombospondins
TTX	Tetrodotoxin
TUNEL	Terminal deoxynucleotidyltransferase-Mediated dUTP-Digoxygenin nick end labelling
UPS	Ubiquitin proteasome system
VAMP	Vesicle associated protein
vGAT	Vesicular γ-aminobutyric transporter
VGCC	Voltage gated calcium channels
vGlut	Vesicular Glutamate transporter

CHAPTER 1:

1. Introduction

Chemical synapses are the fundamental units of neuronal circuits. These highly specialized cellular junctions exist between two adjacent neurons, or a neuron and its target cell, such as a muscle fiber or gland. Critically, synaptic specializations enable unidirectional signal transmission across synapses; action potentials stimulate the release of chemical neurotransmitters from the presynaptic bouton, which are received by specific receptors embedded within the postsynaptic membrane. Neurotransmitter receptors are anchored to complex signaling molecules, which together with ion gated channels, transduce the chemical signaling event back into an electrical potential. Equally critical to neural function is the role of synapses in the summation of synaptic potentials. Postsynaptic cells can only generate action potentials once a postsynaptic potential (PSP) threshold has been met. However, individual synapses only produce minute PSPs, which are insufficient to raise the resting membrane potential and induce postsynaptic firing. A thousand plus synapses, many of which may originate from different neurons, converge to innervate a single neuron, and it is the sum of these PSPs that determine the response of the postsynaptic cell. Therefore, summation ensures that low-level random firing is arrested and only sufficient excitation is propagated. Furthermore, summation integrates excitatory and inhibitory synaptic transmissions. Together, the properties of unidirectional synaptic transmission and synaptic summation allow the integration of stimuli, both temporally and spatially.

In the vertebrate CNS the majority of synapses are stable and the networks they connect are robustly maintained (Holtmaat et al., 2005; Trachtenberg et al., 2002). Critically, this stability underpins core physiological functions such as homeostasis, enteric and cardiovascular function, sensory perception and processing, and the stereotyped motor behaviors that enable us to interact and affect our environments. However, there is also significant turnover of synapses, where populations of synapses assemble and disassemble over time (Goda and Davis, 2003; Holtmaat and Svoboda, 2009). There is now a compelling body of work that strongly suggests experience drives synaptic assembly and disassembly, which together with neurite outgrowth and retraction, underpins neural circuit formation and structural plasticity within the central and peripheral nervous systems (Chklovskii et al., 2004; Feldman and Brecht, 2005). Such dynamic properties of neurites and synapses enable adaptive modifications and the acquisition of new behaviors, for example the acquisition of conditioned fear responses (Dalzell et al., 2010), developing coping strategies to stressful or novel environmental events (Huether, 1998) and translating experience into long-term retrievable memories that mediate behavioral responses (Chklovskii et al., 2004). Evidence also suggests that the co-coordinated formation and elimination of synapses may be a biological construct for higher cognitive processes such as learning and memory (Eaton and Davis, 2003; Holtmaat and Svoboda, 2009).

The delicate balance of synapse assembly, disassembly and maintenance is therefore a critical determinant of health and function within the CNS. Importantly, recent studies suggest that synaptic disassembly may precede or even initiate the massive neurodegeneration that hallmarks devastating diseases such as Alzheimer's, Parkinson's, ALS and glaucoma (Selkoe, 2002; Stevens et al., 2007). The search to understand the mechanisms that regulate and balance synapse assembly, disassembly and maintenance are not only intrinsically interesting for understanding how we perceive and interact with our environment, but also holds the potential for translational research into the nature of neurodegenerative diseases and the ability to develop therapies to facilitate prognosis.

The past two decades have yielded significant insights into synaptic structure and function at the molecular level. Technological advancements in both hardware and molecular manipulations have generated highly sophisticated experimental models. Examples include the generation of conditional mutant animals that enable the study of specific genes at specific developmental stages. Importantly, this approach circumvents embryonic lethality if the gene(s) is required during early development. Combining mutational approaches with fluorescent tagging and imaging technologies has enabled direct, real-time visualization of synapse dynamics within the cortex of rodents to examine how neural connections and circuits respond to environmental changes. In-vitro approaches of cell and tissue culture systems provide researchers with the tools to finely dissect cell-signaling pathways, which have shed light into some of the molecular mechanisms that regulate synapse formation and function. An important finding from genetic and proteomic analyses is how remarkably conserved the synapse is (Kandel, 2001; Ryan and Grant, 2009). Another finding is the extent of redundancy between specific genes products; this may not be too surprising given the essential role of synapses and therefore the need to provide essential safeguards. However, redundancy means that teasing out the molecular mechanisms that regulate synaptic function and dynamics is inherently difficult.

This thesis examines the role of Wnt signaling at the synapse. Secreted Wnt ligands are known synaptogenic factors (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Davis et al., 2008; Farias et al., 2009; Hall et al., 2000; Henriquez et al., 2008; Krylova et al., 2002; Lucas and Salinas, 1997). However, it was not clear whether Wnts act as pan-synaptogenic factors, or whether they preferentially stimulate the assembly of excitatory or inhibitory synapses. The results presented in this thesis contribute to a paper recently published from our lab that demonstrates Wnt7a selectively stimulates the assembly of excitatory synapses without affecting inhibitory synaptogenesis (Ciani et al., 2011). Studies have previously shown that pharmacological inhibition of Gsk3β mimics Wnt-mediated synaptic differentiation (Davis et al., 2008; Hall et al., 2002; Kim and Thayer, 2009). To examine the mechanisms of Wnt-mediated synaptogenesis I further probed the Wnt signaling pathway. I present a model for a divergent canonical-Wnt pathway regulating presynaptic assembly of excitatory synapses in the rat hippocampus. Wnts, and components of the Wnt signaling pathways, are expressed in developing and mature neural tissue (Coyle-Rink et al., 2002; Davis et al., 2008; Gogolla et al., 2009; Lucas and Salinas,

1997; Rosso et al., 2005; Shimogori et al., 2004); this raises the question of whether Wnt signaling plays a role at mature synapses. My analyses from immunohistochemical, electron microscopy and time-lapse imaging, from young and mature hippocampal cultures, demonstrate that acute Wnt-blockade induces significant and rapid synapse disassembly. This implies that endogenous Wnt signaling plays an important role in synaptic maintenance.

Within this introductory chapter I shall, review the current evidence and theories of synaptic structure and function, and the dynamic behavior of synapses in the developing and mature CNS. Wnt signaling will be addressed in detail and how it regulates CNS development and neural circuitry. However, before this review, I shall give a very brief overview on how concepts of neural conductance and transmission have evolved since Aristotle's early philosophical discussions.

1.1. A brief history of the synapse

The idea of a discrete substance being propagated through the body towards muscle fibers to inflict contraction dates back to the 4th Century BC with philosophical discussions by Aristotle (for reviews on the early history of the synapse see (Bennett, 1999; Lopez-Munoz and Alamo, 2009). Indeed, this concept formed the platform to search for physiological constructs of conductance and transmission. In the 2nd and 3rd centuries AD, Galen and his students' refined Aristotle's theories with careful dissections that revealed the brain gives rise to motor nerves that conduct what Aristotle referred to as psychic pneuma (an approximate translation is breath or air of the soul). Galen suggested this substance is transmitted from the nerve terminal to the muscle. An insightful postulation from this group was their speculation that psychic pneuma was a resident property of nerves, and importantly, they described the brain as the organ of the mind. Descartes (1596-1650) a philosopher, mathematician and physicist was next to build upon the concept of neural transmission; he successfully dismantled Aristotle's concepts of psyche and developed theories of conductance and transmission based on mechanical properties, importantly he intimated a flow of particles from the nerve terminal to the muscle.

With the development of light microscopy in the 17th and 18th centuries came descriptions of nerves as hollow tubules filled with liquid substances. This period also saw the emergence of the science of electricity and its biological applications revealed neural conductance as electrical activity. However, it was not until the development of sophisticated histological stains in the early to mid 19th century that neuronal morphology and the structure of nerve endings could be studied. Santiago Ramon y Cajal (1852-1934) indirectly established the existence of synaptic connections in 1892 when he described and vehemently defended his observations from Golgi preparations of freely ending terminal arborizations within the hippocampal neural network (DeFelipe, 2002). Together with degeneration experiments, his work finally settled the controversy of whether neurons anastomose with their target cells to form continuous networks, as dictated by the reticular theory (Cimino, 1999), or whether neurons were discrete cells, which

implicates a form of communication via specialized contacts. Ramon y Cajal's compelling anatomical evidence for the latter gave rise to the neuron doctrine, which is the dogma of modern-day neuroscience.

In the same era, studies from spinal cord preparations by the physiologist Charles Scott Sherrington (1858-1952) led to the conclusion of a "surface of separation" between neurons, and together with colleagues he postulated a "change in the nature of the nervous impulse as it passes from one cell to the other" (for a perspective of Sherrington's research and its impact on modern neuroscience see (Matthews, 1982). Indeed it was Sherrington and colleagues that established the term synapse in the late 1800s, as an amalgamation of "syn", the Greek term for "together" and "haptein", meaning "to clasp". Importantly, when deciding upon appropriate nomenclature, the idea that synapses were active, functional structures was appreciated and was described in correspondences between Sherrington and Sharper-Shafer in 1897, where he wrote "as to junction, I feel we are less reconcilable…the mere fact that junction implies passive union is alone enough to ruin the term…it does not want the gift of prophecy to foretell that it (the term junction) must become more and more inapplicable as research progresses" (Tansey, 1997). This insight would be fully realized within a few decades by the pioneering work of Bernard Katz (1911-2003) who is championed as the founder of the theory for quantal neurotransmitter release as the basis for neural transmission. His colleagues and contemporaries are the giants and founders of modern day neurophysiology and jointly made his groundbreaking work possible. These great scientists include John Eccles, JZ Young, Alan Hodgkins and Andrew Huxley for their inspired research that revealed and measured postsynaptic potentials and the nature of ion channels (Cowan et al., 2000; Eccles, 1982). Together with Otto Loewi and Henry Dale for their landmark contributions in identifying chemical neurotransmission and thus the existence of neurotransmitters (reviewed by (Eccles, 1982). Visual confirmation of synaptic structures were eventually realized in the early 1950s by electronmicroscopy and with this the identification of synaptic vesicles as the putative carriers of neurotransmitter (Luse, 1956; Palay and Palade, 1955). The acknowledgments made here do not give justice to the many extraordinary minds that have contributed to our understanding of the synapse, as this would be a thesis in its own right. The purpose of this small chapter is to highlight seminal periods that shaped the course and evolution of neuroscience as a discipline.

1.2. Synapse structure and function

The specialized structure of the chemical synapse determines unidirectional flow of signal from the presynaptic terminal to the postsynaptic cell. Presynaptic boutons are secretory structures that are structurally organized to transmit chemical messengers; over 1000 proteins cooperate in this primary function, which is triggered by incoming action potentials. Action potentials increase the probability of opening voltage-gated calcium channels, which stimulates synaptic vesicle (SV) fusion with the plasma membrane and consequent release of neurotransmitter into the cleft. The postsynaptic apparatus is equally specialized to fulfill its primary function of signal

transduction. In the case of excitatory synapses, specialized dendritic protrusions, called spines, align with boutons to receive neurotransmitter molecules. Here, receptors are embedded within the plasma membrane and anchored by a complex and dynamic scaffold protein system that connects receptors with ionic gates and downstream effector molecules that initiate multiple signaling cascades. The primary function of the postsynaptic apparatus is to convert chemical interactions back into electrical signals. Therefore, the primary objective of the presynaptic bouton is centered on efficient SV fusion and the release of neurotransmitter, and the postsynaptic terminal is dedicated to neurotransmitter receptor function and dynamics, and the organization of downstream signaling pathways. These fundamental functions are highly conserved and many molecular homologues have been identified from Aplasia and C.elegans to vertebrates.

1.2.1.Presynaptic specialization

Neurotransmitter molecules are contained within SVs, and release occurs at the active zone (AZ), which is a highly specialized and restricted region of the plasma membrane. Synapses vary considerably in their size and general morphology depending on their type, anatomical location and the target cell. However, within this heterogeneity synapses share significant similarities in terms of their structure and function. Ultrastructural studies of the presynaptic compartment reveal three highly conserved morphological features; (1) an organized electron dense plasma membrane, the AZ, which is directly opposed to the postsynaptic density (PSD), (2) a cluster of synaptic vesicles (SVs) within the presynaptic cytomatrix which is associated with the AZ and (3) electron dense projections that extend from the AZ cytomatrix into the cytoplasm, upon which SVs are tethered (Phillips et al., 2001; Zhai and Bellen, 2004). Whilst these three structures may be morphologically distinct, they are intimately linked and cooperate to ensure regulated neurotransmitter release (Dresbach et al., 2001; Phillips et al., 2001; Zamorano and Garner, 2001).

Structure of the active zone and cytomatix at the active zone

The AZ and cytomatrix at the active zone (CAZ) are complex, protein rich structures that regulate SV tethering, fusion and neurotransmitter release. This is effectively achieved through intricate molecular interactions that guide SVs to their fusion site, and conformational changes that alter energy barriers to facilitate membrane fusion (Chen and Scheller, 2001; Jahn et al., 2003; Martens and McMahon, 2008). Furthermore, the active zone engages inter- and extracellular molecules to anchor and stabilize the junction holding it in precise registration.

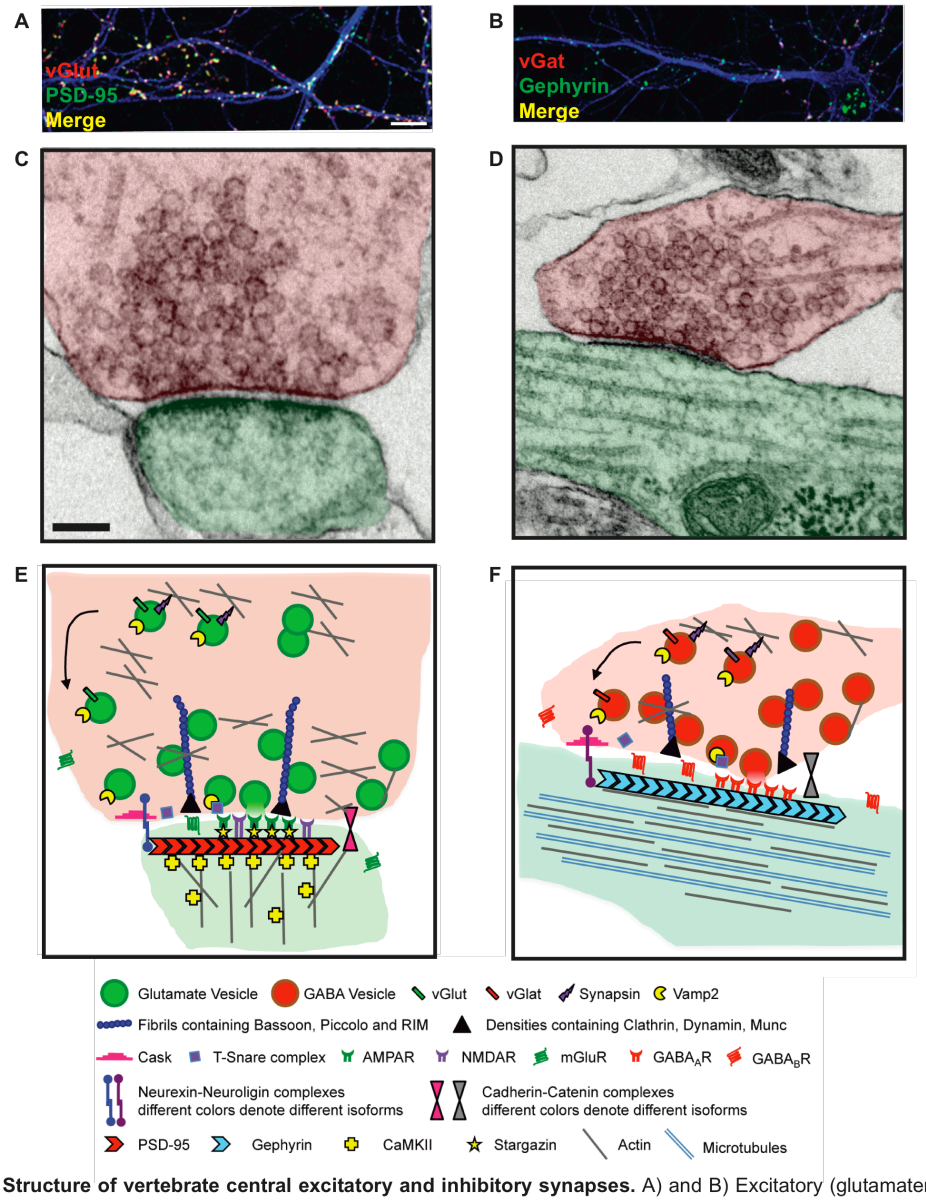


Figure 1.1 Structure of vertebrate central excitatory and inhibitory synapses. A) and B) Excitatory (glutamatergic) and inhibitory (GABAergic) synapses, respectively, in cultured hippocampal neurons (14 DIV) visualized by immunofluorescence. Scale bar 5 µm. A) The majority of vGlut positive presynaptic sites (red), co localize (yellow) with postsynaptic sites containing PSD95 (green) along neurites labelled with antibodies against the cytoskeletal marker Tuj-1 (blue). B) Similarly, many vGAT positive puncta (red) co localise with postsynaptic gephyrin. C) and D) Electronmicrographs reveal structural similarities and differences between excitatory and inhibitory synapses (C and D respectively); presynaptic terminals are structurally similar with clustered synaptic vesicles (SVs) associated with a defined electron dense plasma membrane, referred to as the active zone. Excitatory presynaptic sites appose postsynaptic spines, which contain a defined and thickened electron dense region referred to as the post synaptic density (PSD). In contrast, inhibitory presynaptic terminals synapse directly onto the dendritic shaft, which contain a relatively thin PSD. Scale bar for C) and D) is 0.1µm. E) and F) Cartoons depicting key molecular components of excitatory and inhibitory synapses (E and F respectively). In addition to structural similarities, excitatory and inhibitory presynaptic terminals share many of the same molecules such as; the scaffolding protein Cask; active zone and cytomatrix at the active zone proteins Bassoon, Piccolo, RIM, Munc and T-SNARE complexes. SV associated proteins including Vamp2 and Synapsin1 are also present at both types of synapse. However, the neurotransmitter transporters vGlut and vGAT, which transport glutamate and GABA from the cytoplasm into SVs, are specific to excitatory and inhibitory synapses. Excitatory and inhibitory synapses also contain transynaptic complexes of Neurexin-Neuroligin and Cadherin-Catenin, although the isoforms differ according to synapse type. Postsynaptically, excitatory and inhibitory synapses contain synapse specific ionic and metabotrophic receptors and synapse specific scaffolding proteins.

- *The Presynaptic Web*

Early ultrastructural observations of the AZ revealed an organized webbed structure with electron dense particles arranged in a systematic pattern (Bloom and Aghajanian, 1968; Pfenninger et al., 1972). More recently, electron microscope tomography and 3-D rendering software have produced extraordinary images of AZ and SV configurations at the frog NMJ, where a highly organized array of “beams” and “ribs” join docked SVs with calcium channels (Harlow et al., 2001). At the mammalian central synapse, the AZ exhibits an equally organized system of electron dense particles connected by fibrils (Phillips et al., 2001). These presynaptic densities, which are intimately associated with SVs (Siksou et al., 2007), often appear pyramidal is shape and measure ~50nm in diameter and height (Phillips et al., 2001; Siksou et al., 2007). Fibrils of ~50-100 nm separate the densities and provide docking positions for SVs, which create release sites (Figure 1.2 panel B) (Pfenninger et al., 1972; Triller and Korn, 1985). Careful biochemical analyses of isolated presynaptic webs support the hypothesis that presynaptic densities serve as SV docking and fusion sites (Phillips et al., 2001). Mass spectrometry of presynaptic web fractions has identified proteins involved in SV recycling, cytoskeletal components, adhesion proteins and scaffolding molecules (Phillips et al., 2001). From their progressive fractionation process, the authors postulate the localization of exo- and endocytic machineries within the densities facilitate membrane retrieval from fused SVs for rapid recycling. Furthermore, their data reveal the electron dense fibrils that project from presynaptic densities into the SV pool contain the cytomatrix proteins Piccolo, Bassoon, RIM and Syntaxin. The elegant studies described here demonstrate the remarkable organization of the AZ and exemplifies the intimate relationship between structure and function.

- *Cytoskeletal Associated Proteins*

A number of cytoskeleton and cytoskeleton-associated protein families have been identified within the AZ and CAZ (Figure 1.2 panel B). These can be classified into three functional groups. Firstly, the classical cytoskeletal proteins that form the fundamental framework for the AZ, these include actin, tubulin, myosin, spectrins and β-catenin (Hirokawa et al., 1989; Phillips et al., 2001). Secondly, scaffold proteins such as SAP90/97 and CASK have been identified, which link the cytoskeletal matrix with ion channels and adhesion molecules (Butz et al., 1998; Hata et al., 1996; Hsueh et al., 1998; Maximov et al., 1999). Thirdly, a group of AZ specific protein families have been identified and characterized; these include Munc-13s, RIMs, ELKs, Bassoon and Piccolo, which are all multi-domain proteins that act to spatially and temporally regulate SV tethering, docking, priming and fusion (Dresbach et al., 2003; Hida and Ohtsuka, 2010; Schoch and Gundelfinger, 2006).

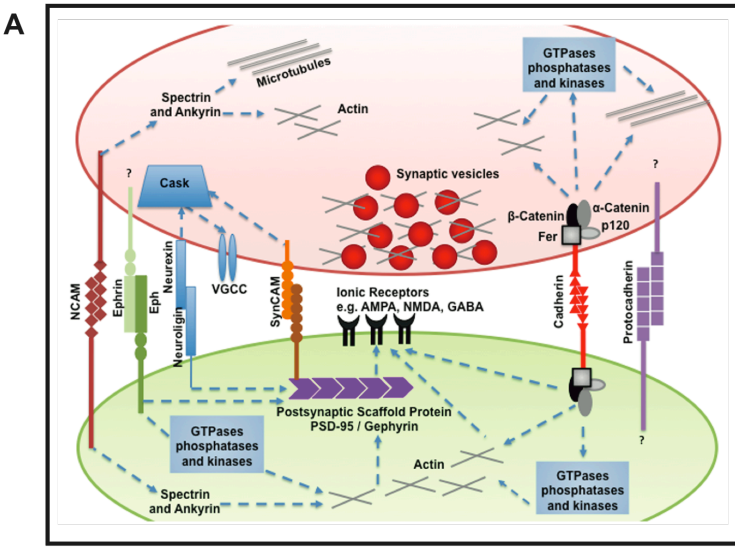
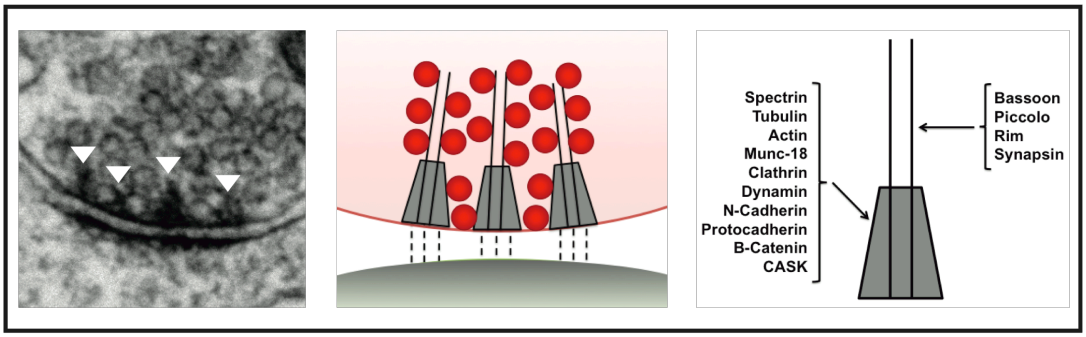
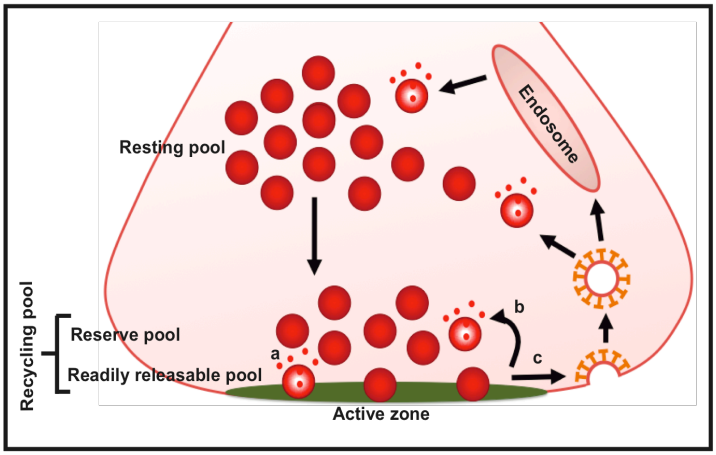


Figure 1.2. Presynaptic molecular structure.
A) CAMs regulate pre and postsynaptic alignment and organization. An array of transynaptic CAMs bridge the synaptic cleft to stabilize the junction and provide a mechanism for bi-directional signaling between the pre- and postsynaptic sites. CAMs such as Cadherins, Eph/ephrins and NCAM regulate cytoskeletal dynamics on both sides of the synapse and/or post-synaptic receptor organization. CAMs such as Neuroligin/Neurexin and SynCAM interact with pre and postsynaptic scaffold proteins, which in turn regulate ionic receptors and channels. Together, CAMs are important regulators of synaptic function, synapse assembly and synaptic maintenance (see text). The depicted arrangement is not spatially accurate or to scale.





B) Model of the presynaptic web. Left panel: electronmicrograph of a synapse containing presynaptic electron dense particles (white arrow heads). The granular appearance of the cleft may represent extracellular CAM domains. Middle and right panels: models illustrating the organization and molecular composition of presynaptic densities. Middle panel depicts electron dense particles with emanating fibrils, which associate with SVs. The densities are spaced (~50nm) to allow individual SVs to dock with the presynaptic plasma membrane. Right panel: The electron dense particles contain molecules involved with adhesion, signaling and endo- and exocytosis. The associated fibrils contain cytomatrix proteins. The cartoons are adapted from Phillips et al. 2001

C) Presynaptic SVs are organized into different functional pools. Vesicles within the readily releasable pool (RRP) are primed and docked at the plasma membrane, ready for exocytosis and neurotransmitter release within milliseconds of stimulation. The reserve pool rapidly replenishes the RRP following stimulation. SVs within the reserve pool are only mobilized in response to high frequency stimulation. Three different models of SV fusion and membrane retrieval from the plasma membrane have been described; a) the “kiss and stay” model describes SVs that fuse with the active zone membrane to release their neurotransmitter cargo, but do not undock. Under this model SVs are rapidly retrieved and remain within the RRP; they are refilled with neurotransmitter at the membrane once the fusion pore has closed. b) The “kiss and run” model describes SVs that undergo partial fusion with the active zone membrane and are exocytosed at the point of fusion. The endocytosed SVs are refilled with neurotransmitter locally and recycled into the reserve pool. c) Clathrin-mediated endocytosis follows full SV fusion and collapse into the active zone membrane. Here, SVs are endocytosed outside of the active zone and are either directly recycled into the resting pool after refueling with neurotransmitter, or passage via an early endosome before refueling and recycling into the resting pool.

Prior to docking SVs are tethered within the CAZ by structural cytomatrix proteins such as Synapsins, Bassoon and Piccolo (Figure 1.2 panel B) (Cases-Langhoff et al., 1996; Hilfiker et al., 1998; tom Dieck et al., 1998). In vertebrate CNS synapses, these proteins are associated with the electron dense projections that emanate from the AZ into the cytoplasm. These projections tether SVs to the AZ (Phillips et al., 2001). Bassoon is a core scaffold protein of the CAZ that is anchored within the CAZ through central domain interactions (Dresbach et al., 2003). Analyses suggest that Bassoon targets SVs via N-terminal sites, probably at the two zinc-finger domains, and the interactions are stabilized through N-myristoylation (Dresbach et al., 2003). The anchoring function of Bassoon was demonstrated by ultrastructural analyses performed in mice deficient in Bassoon (Dick et al., 2003). Here, synaptic ribbons within retinal photoreceptor cells, which are equivalent to the electron dense projections of the CAZ, were clearly dissociated from the AZ (Dick et al., 2003). Importantly, this correlated with impaired synaptic transmission (Dick et al., 2003).

- *Cell-Adhesion Molecules*

Once SVs have released their cargo, neurotransmitter molecules diffuse across the synaptic cleft, which is typically ~25nm wide, to bind with receptors embedded within the postsynaptic membrane. Therefore, the AZ and PSD must be precisely aligned and the junction must be maintained in a relatively stable state. Presynaptic cell-adhesion molecules (CAMs) bind with postsynaptic partners or the extracellular matrix (ECM) (Figure 1.2 panel A). Examples of CAMs characterized at the synapse include neurexins (Craig and Kang, 2007; Dean and Dresbach, 2006; Lise and El-Husseini, 2006), neural cell adhesion molecules (NCAMs) (Rougon and Hobert, 2003; Shapiro et al., 2007), SynCAM (Biederer et al., 2002; Fogel et al., 2007), cadherins (Shan et al., 1999; Yagi and Takeichi, 2000), protocadherins (Frank and Kemler, 2002; Morishita and Yagi, 2007) and integrins (Chavis and Westbrook, 2001). Presynaptic CAMs contain extracellular domains that bind with their postsynaptic partners to form homo- or heterophillic interactions that span the synaptic cleft. Indeed, electron dense projections extending from the AZ and PSD into the synaptic clef have been observed in electron micrographs (Ichimura and Hashimoto, 1988), plus my own observations (Figure 1.2 panel B).

In addition to transynaptic adhesive properties, some CAMs contain intracellular domains that bind to scaffold proteins in the pre and/or postsynaptic side to anchor and stabilize the synapse (Figure 1.2 panel A). For example, presynaptic neurexins (NX) bind with the scaffold protein CASK within the AZ; and its postsynaptic partner, neuroligin (NL), binds to the major postsynaptic scaffold protein PSD-95 (Missler and Sudhof, 1998; Sheng and Sala, 2001). Similarly, SynCAM, which is present on both sides of the synapse (Biederer et al., 2002; Fogel et al., 2007) binds with CASK and PSD-95 (Fogel et al., 2007). Some CAMs also interact with effector molecules to initiate signaling cascades, for example the cadherin-catenin complex regulates activity of small GTPases Rho and Rac as well as protein phosphatases and kinases (Goodwin and Yap, 2004). The cadherin-catenin complex also regulates F-actin and microtubule-associated proteins resulting in changes in cytoskeletal dynamics and synapse

morphology and function (Arikkath and Reichardt, 2008; Bamji, 2005; Dillon and Goda, 2005; Kwiatkowski et al., 2007; Tai et al., 2008).

Synaptic vesicles and neurotransmitter release

SVs are uniformly spherical secretory organelles (~40nm diameter) and their population size within presynaptic boutons varies tremendously between synapses; in the mammalian hippocampal synapse it typically ranges from 30 to several hundred vesicles (Schikorski and Stevens, 1997). However, only 2 to 8 SVs are typically docked at each AZ at any one time (Satzler et al., 2002; Schikorski and Stevens, 2001). Interestingly, the number of docked vesicles does not correlate strongly with the size of the total SV population, but does correlate with the size of the PSD (Siksou et al., 2007).

- *Synaptic Vesicle Pools*

Presynaptic SV populations are organized into different pools, the resting pool and the recycling pool (Figure 1.2 panel C). The concept of different SV pools was developed from measuring different release rates under various stimulation conditions. The release of neurotransmitter from individual SVs is referred to as quantal release and is defined electrophysiologically as individual amplitude peaks. The recycling pool refers to all the SVs that participate in exo- and endocytosis during prolonged stimulation (Schweizer and Ryan, 2006; Sudhof, 2004), and is further divided into the reserve pool and the ready releasable pool (RRP) (Figure 1.2 panel C). The RRP is physiologically defined as the pool of SVs that immediately release neurotransmitter in response to elevated intracellular Ca^{++} or stimulation (Rosenmund and Stevens, 1996; Schneggenburger et al., 1999). These vesicles were interpreted as the docked SVs visualized in electronmicrographs, together with fusion competent SVs, referred to as “cocked” vesicles (Sudhof, 1995). Following high frequency stimulation the release rate drops significantly until it reaches a new low steady-state level; the initial depression reflects depletion of SVs from the RRP and the steady state level probably reflects deployment of SVs from the reserve pool. (Rosenmund and Stevens, 1996; Satzler et al., 2002; Schikorski and Stevens, 2001). In cultured hippocampal neurons, the size of the reserve pool is ~17-20 SVs, as measured by FM1-43 labeling of SVs (Murthy et al., 1997). However, the notion of discrete SV pools, as a theory for all synapses is problematic, as considerable discrepancies have arisen in calculations of quantal release and the estimated releasable pool size from measurements taken at the calyx of Held and the vertebrate NMJ (Pan and Zucker, 2009; Sudhof, 2004). Nevertheless, the distinction of SV pools based upon their participation in release is broadly accepted and further research and development of models will elucidate the nature of SV populations and how they contribute to release probability.

- *Synaptic Vesicle Exo- and Endocytosis*

Fast neurotransmission is a calcium dependent process that relies upon rapid recycling of SVs within the presynaptic bouton. To achieve this a subpopulation of SVs are tethered to the AZ where they are primed to become fusion-competent; the trigger for final fusion is an increase in intracellular calcium concentration. Action potentials increase the probability of voltage-gated calcium channels opening. Only a small proportion of tethered SVs are primed at any one time and the size of this population, the RRP, determines synaptic release probability and therefore the capacity for neural conductance (Sudhof, 1995; Zucker, 1996).

SNARE Proteins and membrane fusion

SV fusion with the presynaptic plasma membrane is enabled and regulated by complexes of synaptic SNARE (Soluble NSF attachment protein receptors) proteins. The most characterized SNAREs are synaptobrevin/VAMP2 on the SV plasma membrane and Syntaxin and SNAP25 within the AZ (Bennett et al., 1992; Malsam et al., 2008; Oyler et al., 1989; Sorensen, 2009; Sudhof et al., 1989; Sudhof and Rothman, 2009). The SNARE hypothesis was originally developed from affinity purification studies and binding assays (Sollner et al., 1993). These experiments isolated proteins within SVs (v-SNARES) and the AZ (t-SNARES) and the data suggested a simple docking mechanism between the two SNARE protein groups. More recently, researchers have identified precise combinations of SNARES and have described how the molecular structures combine to form tight coiled-coil complexes (Malsam et al., 2008; Rettig and Neher, 2002; Sudhof and Rothman, 2009). These conformations draw the two membranes together to exert mechanical forces and liberate the necessary energy required to overcome the energy barrier of lipid rearrangements, the process that drives membrane fusion (Malsam et al., 2008; Rettig and Neher, 2002; Sudhof and Rothman, 2009).

Genetic studies in C. elegans (Richmond et al., 1999), Drosophila (Aravamudan et al., 1999) and mice (Augustin et al., 1999; Varoqueaux et al., 2002) have shown that the conserved Munc-13/UNC13 proteins are essential for SV priming. Functional analyses suggest that Munc-13 binding with the t-SNARE protein syntaxin promotes a loose SNARE complex (Betz et al., 1997). Munc-13 is positively regulated by interactions with RIM1, which binds with SV associated Rab3 proteins (Betz et al., 2001); these interactions form a tripartite Rab3-RIM1-Munc13 scaffold complex, which brings SVs into close proximity with the priming and fusion machinery (Dulubova et al., 2005). Importantly, RIM1 also interacts directly with the t-SNARE protein SNAP25, Synaptotagmin1, which is a SV associated calcium sensor protein, and voltage gated calcium channels (Coppola et al., 2001); in this complex formation SVs are primed and ready for membrane fusion.

Synaptotagmin1 also binds with the plasma membrane associated protein Complexin, and this critical interaction prevents final fusion (Rizo and Rosenmund, 2008). Therefore, interactions between Complexin and Synaptotagmin1 clamp the fusion machinery and hold it in a primed state that needs minimal triggering (Sudhof and Rothman, 2009). When Ca^{++} enters the

presynaptic bouton or terminal, as a result of action potential firing, it binds with Synaptotagmin1, which releases the Complexin hold and final fusion can proceed. The molecular interactions and events described here therefore enable tight regulation of SV exocytosis, which is required for neurotransmitter release and organized information processing within the CNS.

- *Models of synaptic vesicle recycling*

Synaptic vesicle recycling is an efficient, localized system that ensures sustained release probability. Following exocytosis, vesicles are endocytozed. Currently, three different pathways are described and controversy persists as to whether different models apply to different stimulation conditions. The three pathways are 1) the kiss-and-stay model, 2) the kiss-and-run model and 3) endocytosis via clathrin mediated-pits (Figure 1.2 panel C) (Galli and Haucke, 2001; Gundelfinger et al., 2003; Sudhof, 2000). In all cases the retrieved SV are re-filled with neurotransmitter through the use of specific neurotransmitter transporters. Under the kiss-and-stay model, SVs do not "undock" once the cargo is released, rather they remain within the RRP and are re-filled at the membrane (Pyle et al., 2000). In the second model, fused SVs are endocytozed at the point of fusion and are refueled locally and return to the recycling pool (Aravanis et al., 2003; Klyachko and Jackson, 2002; Sun et al., 2002). Alternatively, SVs undergo full collapse and their membranes are integrated into the plasma membrane at the AZ. Endocytosis then occurs at regions adjacent to the AZ via clathrin-mediated pitts, and SV refueling is either local or via an early endosomal intermediate before entry into the reserve pool (Slepnev and De Camilli, 2000). The immediate difference between these routes is the speed at which SVs are recycled and become available for release. It has been suggested that all the models are valid and indeed may occur simultaneously (Sudhof, 2004). Alternatively, the fast or slow track route may be preferentially utilized depending on the type of synapse, the type or size of stimulation or the SV pool mobilized and the number of docked SVs (Galli and Haucke, 2001). As yet, the precise details of how SVs are recycled, and whether different synapses employ different pathways, have yet to be fully understood.

The actin cytoskeleton in presynaptic structure and function

Imaging studies reveal that GFP-actin surrounds and co-localizes with SV pools at central synapses, where it acts as a scaffold structure to cluster and anchor SVs in position and also segregate the RP and RRP (Morales et al., 2000; Sankaranarayanan et al., 2003). F-actin associates with Synapsin1 (Landis et al., 1988). Synapsins are a group of highly conserved neuronal phosphoproteins that reversibly associate with actin, microtubules, spectrin and SVs in a phosphorylation dependent manner (Bahler and Greengard, 1987; Benfenati et al., 1989; Benfenati et al., 1993; Ceccaldi et al., 1995; Cesca et al., 2010; De Camilli et al., 1990; Greengard et al., 1993; Hosaka et al., 1999). Neuronal activity alters the phosphorylation state of Synapsin1a, which weakens interactions between SVs and actin and liberates SVs for

mobilization into the recycling pool for fusion (Fdez and Hilfiker, 2006). Actin filaments also provide a molecular track to guide SVs to the RRP to replenish the RRP during stimulation (Sakaba and Neher, 2003) via actin motor proteins in a calcium-dependent manner (Prekeris and Terrian, 1997).

Actin dynamics also regulate SV recycling. Disruption of F-actin by latrunculin treatment is associated with an increase in the rate of exocytosis, as measured by FM1-43 labeling (Sankaranarayanan et al., 2003) and an increase in mEPSCs (Morales et al., 2000). These studies are supported by genetic studies from LIMK-1 knockout mice that are defective in actin depolymerisation (Meng et al., 2002). Together, these studies suggest F-actin negatively regulates exocytosis, possibly through interactions with the fusion machinery (Dillon and Goda, 2005).

In contrast, F-actin positively regulates clathrin-mediated SV endocytosis by facilitating the pinching off of SVs from the endocytic zone (Engqvist-Goldstein and Drubin, 2003). F-actin provides a structural framework for guiding newly endocytosed SVs back to the RP (Shupliakov et al., 2002). The actin cytoskeleton therefore provides structural boundaries to contain SVs within the presynaptic compartment and also provides the necessary molecular guides that organize SV mobilization and retrieval during SV recycling.

1.2.2. Postsynaptic differentiation

Unlike the presynaptic bouton, postsynaptic morphology varies considerably depending on the type of synapse. Early ultrastructural studies revealed two categories of synapse, type 1 and type 2 (Gray, 1959), which were later described as asymmetric and symmetric synapses (Colonnier, 1968). Asymmetric synapses are found primarily on dendritic spines and are distinguished by a thickened PSD, whereas symmetrical synapses are found on dendritic shafts or at the cell body and do not contain a prominent PSD (Figure 1). These two types of synapses are now known to be excitatory and inhibitory synapses respectively. The core function of postsynaptic sites is signal transduction from neurotransmitter receptor binding. The downstream signaling events either activate or inhibit the conversion of chemical signaling into electrical impulses. This is principally achieved by an array of transmembrane ionic and metabotrophic receptors, which are anchored by complexes of scaffold proteins that also act to localize signaling molecules within the postsynaptic compartment. Historically, much of the research dedicated to central postsynaptic signal transduction has focused on glutamatergic dendritic spines, which are the principle sites for excitatory synaptic transmission in the brain. It is only in more recent years that detailed analyses of inhibitory structure and function has advanced.

Excitatory postsynaptic differentiation

Dendritic spines are small protrusions that stem from the dendritic shaft and are the primary postsynaptic sites for excitatory transmission. A characteristic of spines is a constricted neck region (Bourne and Harris, 2008), which provides a discrete biochemical compartment to synchronize activation of glutamate receptors with calcium regulation and complex signaling cascades, cytoskeletal remodeling, local receptor trafficking and protein synthesis and degradation (Bourne and Harris, 2008; Newpher and Ehlers, 2009). All spines contain a PSD, which is visualized in electronmicrographs as an electron dense region associated with the postsynaptic membrane. The PSD is composed of many hundreds of proteins, of which the core scaffold protein is PSD-95 (Collins et al., 2006; Okabe, 2007). PSD-95 contains multiple domains including three conserved PDZ domains, an SH3 domain and a guanylate-kinase like domain (Kim et al., 2007; Scannevin and Huganir, 2000). These functional domains mediate the stabilization of nascent spines and promote synapse maturation and strengthening (El-Husseini et al., 2000; Elias et al., 2006), in part by recruiting and anchoring receptors and other scaffolds at the synapse (Ehrlich et al., 2007; Kim and Sheng, 2004; Kim et al., 2007; Marrs et al., 2001; Okabe et al., 2001a).

The diversity and complexity of signaling cascades within dendritic spines is immense. The key ionic receptors at glutamatergic synapses are AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) and NMDA (N-methyl-d-aspartate) receptors (AMPA-R and NMDA-R respectively). These tetrameric receptors exist in multiple subunit configurations, with different permutations conferring different functional properties, such as cation permeability and gating kinetics (Cull-Candy et al., 2001; Greger et al., 2007; Madden, 2002; Paoletti and Neyton, 2007; Wollmuth and Sobolevsky, 2004). AMPA-Rs are principally selective to Na^+ and K^+; glutamate binding induces rapid conformational changes that enable regulated cation influx, which in turn depolarizes the postsynaptic membrane. NMDA-Rs are principally permeable to Ca^{++}, but only once the membrane has been depolarized through AMPA-R activation. NMDA-Rs contain a Mg^{++} binding site within the channel pore; hyperpolarizing potentials drive extracellular Mg^{++} into the receptor pore, which blocks cation permeability. Membrane depolarization releases the Mg^{++} ion enabling cation flow. This requisite means that NMDA-Rs act as coincidence detectors to regulate postsynaptic signal transduction. Indeed, electrophysiologically “silent” synapses, which are prevalent in early postnatal development, only contain NMDA-Rs (Kerchner and Nicoll, 2008; Petralia et al., 1999). High frequency stimulation stimulates NMDA-R only synapses to recruit and insert AMPA-Rs into the PSD and postsynaptic membrane (Ehlers, 2000; Isaac et al., 1995; Kopec et al., 2006; Liao et al., 1995; Liao et al., 1999; Petralia et al., 1999). AMPA-Rs are trafficked in and out of the PSD laterally and via endo- and exocytic pathways (Groc and Choquet, 2006; Makino and Malinow, 2009; Park et al., 2004). In comparison, NMDA-Rs are relatively stable and remain integrated within the PSD. These distinctive behaviors, which are regulated by differential interactions with PSD scaffold proteins, ultimately determine the level of postsynaptic depolarization (Sheng and Sala, 2001).

Intracellular signaling through calcium/calmodulin-dependent-kinase II (CaMKII) is one of the most characterized signaling pathways in the spine due its central role in synaptic plasticity (Lisman et al., 2002; Sanhueza et al., 2007). CaMKII is highly enriched in the PSD (Kennedy et al., 1983) and its translocation there is stimulated by NMDA-R Ca^{++} influx (Shen and Meyer, 1999; Shen et al., 2000). CaMKII phosphorylates the PSD scaffold protein Stargazin, which regulates AMPA-R trafficking and anchoring to the PSD (Tomita et al., 2005). Furthermore, CaMKII directly phosphorylates the GluR1 subunit of AMPA-Rs to increase channel conductance (Andrasfalvy and Magee, 2004; Barria et al., 1997; Derkach et al., 1999; Luthi et al., 2004). CaMKII signaling also extends to actin dynamics, which directly affect spine morphology. CaMKII directly interacts with filamentous polymers (F-actin) filaments (Honkura et al., 2008; Okamoto et al., 2007; Shen et al., 1998) and therefore may provide a critical link to couple spine morphology and function (Okamoto et al., 2009).

- *Spine morphology and plasticity*

Spine morphologies vary considerably, yet within this heterogeneity there are distinct morphological categories. Cortical spines measure typically measure between 0.015-0.77μm^3 (Knott et al., 2006) and form long, thin structures to short and stubby, or mushroom shaped or branched (Bourne and Harris, 2008). Whilst the relationship between spine morphology and function is not fully understood, there is general consensus that morphology relates to function (Kasai et al., 2003; Yuste and Bonhoeffer, 2001). For example, there is a positive correlation between spine size, PSD area, receptor content, and excitatory postsynaptic potentials (EPSPs) (Matsuzaki et al., 2001). Furthermore, there are strong correlations between the size of the PSD and spine head with the presynaptic AZ, and SV population (Harris et al., 1992; Harris and Stevens, 1989), which suggests coordinated pre and postsynaptic structural modifications (Lisman and Harris, 1993).

Spines are highly plastic structures that undergo morphological changes in an activity-dependent manner (Alvarez and Sabatini, 2007; Bourne and Harris, 2008; Holtmaat and Svoboda, 2009). Long-term *in-vivo* imaging of the barrel cortex in GFP-expressing mice reveals that sensory deprivation, through whisker trimming, induces a loss of spine volume and synapse elimination (Holtmaat et al., 2006). Spine shrinkage and elimination, under this paradigm, is associated with the formation of new spines, which grow in volume as they become stabilized (Knott et al., 2006). Similar morphological spine changes occur in the motor cortex in response to the acquisition of novel motor skills (Xu et al., 2009). Forelimb-reaching tasks in mice induce rapid spine formation (within one hour), which become enlarged and stabilized during subsequently training episodes. Importantly, these spine dynamics are associated with behavioral improvements (Xu et al., 2009).

In-vitro studies corroborate *in-vivo* activity-dependent spine dynamics and adaptations. Long-term potentiation and long-term depression (LTP and LTD respectively) are the most established and best characterized cellular models for neural plasticity and are induced by

electrical or chemical stimuli (Bliss and Lomo, 1973; Dudek and Bear, 1992; Malenka and Bear, 2004; Mulkey and Malenka, 1992). LTP induces the formation of *de novo* spines and persistent increases in spine volume in existing spines (Kopec et al., 2006; Matsuzaki et al., 2004). Conversely, LTD induces persistent loss of spine volume and complete spine elimination (Becker et al., 2008; Nagerl et al., 2004; Zhou et al., 2004). Importantly, LTP and LTD are associated with increases and decreases in synaptic strength respectively, and accordingly, correlated changes in postsynaptic glutamate receptor content (Malenka and Bear, 2004).

- *The actin cytoskeleton in spine structure and function*

F-actin is the major cytoskeletal component of dendritic spines (Cohen et al., 1985; Fifkova and Delay, 1982; Landis and Reese, 1983) and evidence from genetic and pharmacological studies suggest that actin dynamics drives spinogenesis, spine stability and function (Cingolani and Goda, 2008; Halpain, 2000; Matus, 2000). These critical roles are regulated by different pools of actin; fluorescence resonance energy transfer (FRET) with two-photon laser scanning microscopy has revealed tetanic stimulation significantly alters the equilibrium status between the monomeric “globular” form of actin (G-actin) and F-actin (Okamoto et al., 2004). Glutamate un-caging induces localized actin polymerization, which is associated with spine head expansion and elevated AMPA-R mediated currents through activation of NMDA-Rs and CaMKII (Matsuzaki et al., 2004). Importantly, the addition of F-actin into spines provides further binding capacity for receptors and signaling molecules (Okamoto et al., 2004). Actin polymerization stimulates CaMKII recruitment into spines (Okamoto et al., 2004) and also regulates AMPA-R trafficking, through interactions with linker proteins, in an activity-dependent manner (Malinow and Malenka, 2002). Blockade of F-actin polymerization by latrunculin-A treatment, significantly reduces the clustering AMPA-Rs, NMDA-Rs and CaMKII and blocks LTP maintenance induced by high frequency stimulation (Krucker et al., 2000). Furthermore, depolymerization of F-actin, through low frequency stimulation, induces significant spine head shrinkage (Okamoto et al., 2004). Together, these studies demonstrate that actin dynamics are regulated by activity and serve to mediate spine structure, function and plasticity.

The cohort of signaling molecules that affect actin dynamics at the synapse is extensive. Actin dynamics are directly mediated by AMPA-R and NMDA-R activation (Cingolani and Goda, 2008) and by transynaptic complexes of Eph/Ephrin via Rho and Ras GTPases (Irie and Yamaguchi, 2002). Rho GTPases such as Cdc42, Rac and RhoA modulate actin dynamics via downstream effector molecules such as Profilin, Cortactin, Drebrin and Arp2/3 (Hall, 1998; Hotulainen et al., 2009; Kennedy et al., 2005; Rex et al., 2009; Wegner et al., 2008). During synaptic spine-head development Arp2/3 regulates actin polymerization, nucleation and the formation of a branched actin network, whereas coffin is proposed to regulate the length of actin filaments to prevent irregular protrusions out of the spine head (Hotulainen et al., 2009). Coratctin is an F-actin binding protein that regulates cross-linking and actin dynamics (Huang et al., 1997), and is enriched within the PSD where it binds with the PSD scaffolding protein Shank in an activity-dependent manner (Naisbitt et al., 1999).

The cadherin/catenin adhesion complex also interacts with the actin cytoskeleton at the synapse (Bamji, 2005; Kwiatkowski et al., 2007). Genetic studies from mice have shown that N-Cadherin is required for spine stability, probably through its C-terminal domain, which interacts with F-actin (Abe et al., 2004). In addition, α- and β-Catenins, which binds to the cytoplasmic tail of N-Cadherin in both the pre- and postsynaptic compartments, also signal to the actin cytoskeleton (Gates and Peifer, 2005). N-Cadherin and Eph/ephrin are therefore well poised to modulate actin dynamics on both sides of the synapse, and further research will elucidate whether activity co-ordinates pre- and postsynaptic actin dynamics through transynaptic complexes.

In summary, the specialized structures of dendritic spines provide discrete compartments that integrate biochemical events that affect receptor dynamics and morphological changes, which together confer postsynaptic plasticity. Critically, plasticity is a defining feature of an adaptive neural system. Whilst research has identified many of the molecular mechanisms that regulate spine structure and dynamics, it is still not clear how different signaling pathways converge on receptors, their scaffolds or associated proteins. Similarly, how the many signaling pathways integrate to regulate cytoskeletal dynamics remains poorly understood.

Inhibitory postsynaptic differentiation

Inhibitory synapses are vital components of the CNS neural circuitry as they spatially and temporally regulate and integrate neural activity (Spruston, 2008). In contrast to excitatory spines, inhibitory postsynaptic sites are relatively unspecialized; they form directly on to the dendritic shaft and do not possess discrete compartments for restricted biochemical signaling (Figure 1). The predominant sites of synaptic inhibition in the brain are GABAergic synapses. GABA (ϒ-aminobutyric acid) acts on two receptors, $GABA_A$ and $GABA_B$, both of which are selectively permeable to Cl^-. $GABA_A$ receptors ($GABA_A$–R) are fast acting ionic receptors, whereas $GABA_B$-Rs are relatively slow acting metabotrophic receptors that act through second messengers (Farrant and Nusser, 2005). As with Glutamate receptors, the specific subunit composition of GABA-Rs determines ionic conductance and gating kinetics (McKernan and Whiting, 1996). The influx of anions via GABA-Rs causes hyperpolarization of the postsynaptic plasma membrane, which reduces the probability of the postsynaptic cell firing action potentials. Therefore, inhibitory synapses directly affect summation and can inhibit neural transmission.

The PSD at inhibitory synapses, which is considerably thinner compared to excitatory PSDs, is primarily composed of the scaffold protein Gephyrin (Fritschy et al., 2008) (Figure 1). Like PSD-95, Gephyrin contains multiple functional domains and forms a network to anchor receptors and signaling molecules, as well as interactions with the actin cytoskeleton (Jacob et al., 2005; Jacob et al., 2008; Kneussel and Loebrich, 2007; Loebrich et al., 2006; Thomas et al., 2005). These interactions are dynamic and enable a constant turnover of scaffold, receptors and associated proteins, a mechanism that underscores receptor trafficking and recycling, and

consequently postsynaptic strength and plasticity (Feldman, 2009; Kittler et al., 2000; Kneussel and Loebrich, 2007; Kravchenko et al., 2006; Maas et al., 2006; Tyagarajan and Fritschy).

1.2.3.Inhibitory and excitatory neural circuits in the CNS

The development and function of neural circuits depend critically upon a delicate balance between excitatory and inhibitory transmission. Excitatory and inhibitory transmission in the brain is predominantly mediated through glutamate and GABA respectively. The quantity and distribution of glutamatergic and GABAergic inputs on to a single neuron impacts significantly on its output, this in turn determines circuit formation and plasticity (Gulledge et al., 2005). Pyramidal neurons have been extensively studied morphologically and physiologically, and their capacity for integrating excitatory and inhibitory inputs, from local and distant neurons, underscores neural computation (Spruston, 2008). Pyramidal neurons receive different synaptic inputs onto distinct domains; local Glutamatergic inputs are generally made onto proximal dendrites and distant Glutamatergic inputs are predominantly on distal dendritic tufts (Spruston, 2008). GABAergic inputs are mainly perisomatic but significant numbers are also distributed throughout the dendritic arbor (Beaulieu et al., 1992; Bourne and Harris, 2010; Liu, 2004; Miles et al., 1996). The presence of distal inhibitory inputs raises the possibility of regional inhibition mediating plasticity within dendritic segments, which would endow finer control and integration of neural activity (Bourne and Harris, 2010; Spruston, 2008).

In-vitro studies have demonstrated that LTP induction via theta-burst stimulations (TBS) induces coordinated changes in the size and distribution of both excitatory and inhibitory synapses (Bourne and Harris, 2010). Analyses of synapse number and PSD surface area along dendritic segments reconstructed in 3-dimensions from serial transmission electron micrographs reveal that 2-hours after TBS there is a significant loss of small excitatory and inhibitory synapses with a parallel increase of PSD area within the remaining synapses. These findings suggest that a mechanism exists for regulating and coordinating excitatory and inhibitory transmission (Bourne and Harris, 2010). However, the molecular nature of such mechanisms has yet to be elucidated.

In the healthy brain, the ratio of excitatory and inhibitory inputs determines the capacity for sensory processing, cognitive function, decision-making, motor control and sleep (Cline, 2005). A relatively common example of imbalance between excitatory and inhibitory transmission in the brain is epilepsy. There are many forms of epilepsy and the underlying etiologies are complex and varied, and in many cases remain poorly understood (Cope et al., 2009; Crunelli and Leresche, 2002; Epilepsy, 1989; McCormick and Contreras, 2001). However a common pattern of brain activity recorded by electroencephalograms (EEG) from epileptic patients during seizure is a disproportionate and runaway shift towards excitatory transmission (Brenner, 2004). Many antiepileptic drugs potentiate GABAergic transmission in an attempt to balance the excessive excitation that triggers seizures. Clinical studies have shown children born of pregnant women treated with such drugs are of increased risk to impaired intellectual and psychomotor development (Scolnik et al., 1994). Mouse models using the same drugs show

that early postnatal administration significantly impairs reflex development as well as learning and memory in the adult (Levav et al., 2004). These studies show that excessive inhibition within the nervous system during development can have devastating and long-term effects on the function of neural circuits. Imbalances of excitation and inhibition are also understood to underlie neuropathological disorders such as autism (Munoz-Yunta et al., 2008; Rubenstein and Merzenich, 2003), Tourettes Syndrome (Singer and Minzer, 2003), schizophrenia (Kehrer et al., 2008) and Parkinson's disease (Llinas et al., 1999). Therefore, understanding how the delicate balance of excitation and inhibition is established during development and is how it is maintained throughout life is vitally importance to understanding how neural circuits integrate.

Regulation of inhibitory and excitatory synapses

As previously described, intracellular Cl^- is a key determinant of excitation and inhibition in the CNS. Developmental expression of the Cl^- transporter proteins NKCC1 and KCC2 regulate intracellular Cl^- levels (Ben-Ari, 2002). NKCC1 is a sodium-potassium-chloride co-transporter, which pumps Cl^- into the cell and is expressed early in neurons. Conversely, KCC1, which is a potassium-chloride co-transporter, transports Cl^- out of the cell and is expressed in more mature neurons (Ben-Ari, 2002). The developmental expression pattern of these specific transporter proteins therefore has the capacity to determine the ionic driving force, and consequently the reversal potential for $GABA_AR$ mediated responses (Fiumelli and Woodin, 2007). A number of studies have shown that neurons initially receive GABAergic inputs before glutamatergic (Akerman and Cline, 2006; Deng et al., 2007; Tyzio et al., 1999). Importantly, this sequential innervation also occurs in the adult hippocampus whereby newly generated neurons exhibit depolarizing GABAergic inputs before glutamatergic input (Ge et al., 2006). These data suggest a common principle for establishing early circuitry. Indeed, evidence suggests that depolarizing GABAergic transmission is necessary for the formation of glutamatergic synapses, possibly by $GABA_AR$ facilitating NMDA-R activation (Anderson et al., 2004; Ben-Ari, 2002; Ben-Ari et al., 1997; Lee et al., 2005). In turn, the establishment of excitatory glutamatergic transmission may regulate the development of inhibitory GABAergic inputs in an activity dependent manner (Bourne and Harris, 2010; Liu, 2004). Whilst evidence supports this sequence of events, the molecular mechanisms have yet to be elucidated.

Compelling evidence now exists for the role of neurexin-neuroligin (NXNL) isoforms as important determinants of excitatory or inhibitory synapse identity. These heterophyllic transynaptic adhesion molecules were initially characterized as synaptogenic factors (see next chapter). Endogenous NL-1 and -2 preferentially localize to excitatory and inhibitory synapses respectively (Song et al., 1999; Varoqueaux et al., 2004), although some endogenous NL2 is present at excitatory synapses, the proportion decays as synapses mature. Importantly, overexpression of each isoform enhances the number of each respective type of synapse.

Alternative splicing of NL-1 and -2 regulates NL function by mediating NL interactions with different scaffold protein partners, and also binding with counterpart NXs (Chih et al., 2006). For

example, alternatively spliced residues within the LNS domain of NX-1β determines selective clustering of NL-1, -3 and -4 to different degrees, as well as the clustering of Glutamatergic postsynaptic proteins, but do not affect the behavior of NL2 clustering or the molecular composition of inhibitory synapses (Graf et al., 2006). Extensive mutation analyses using constructs in co-culture systems have identified that NX instructs postsynaptic differentiation and specification in a calcium dependent manner, which strongly suggests that NX acts through activity-dependent mechanisms (Graf et al., 2006).

Overexpression studies in cultured hippocampal neurons reveal ectopic PSD95 enhances recruitment of NL1 to nascent postsynaptic sites, and *visa versa* ectopic NL1 increases PSD95 recruitment (Chih et al., 2005; Levinson et al., 2005; Prange et al., 2004). These findings suggest that PSD95 and NL1 can reciprocally target one another to postsynaptic sites *in-vitro*. Furthermore, overexpression of PSD95 alters the localization of NL2 from inhibitory to excitatory synapses (Graf et al., 2004; Levinson et al., 2005). Conversely, knockdown of PSD95 with siRNA moves NL1 from excitatory to inhibitory sites (Gerrow et al., 2006). This seemingly promiscuous behavior of NLs is determined through their C-terminal PDZ binding domains (Levinson et al., 2010); NL-1 and -2 can interact with both PSD-95 and Gephryn, and it is posited that their correct localization is determined by favorable binding affinities (Levinson et al., 2005), possibly determined by alternative splicing. Evidence of other functional domains within the cytoplasmic NL tail suggests that adaptor proteins may also play a role in regulating NL-scaffold protein interactions and their appropriate targeting (Dresbach et al., 2004; Levinson et al., 2010); however, their identification has yet to be determined.

NX-NL isoform interactions are the most characterized molecules for regulating the formation, maturation and balance of excitatory and inhibitory synapses, but their story is incomplete. Further studies are required to fully characterize the temporal order of recruitment of NXs, NLs and scaffolding proteins to nascent synaptic sites. In addition, understanding the binding affinities between different NL isoforms and specific scaffolds and adaptor proteins will shed light on how NLs are targeted to specific synaptic sites. It is also important to understand how NX-NL complexes interact with other adhesion molecules, such as SynCam, cadherins and Eph/ephrins, and also whether they interact with signaling cascades initiated by secreted synaptogenic factors such as BDNF, FGFs and Wnts.

1.2.4.Summary of synapse structure and function

The structure to function relationship that has motivated biological research through the ages is exemplified at synapses; electronmicrographs reveal highly structured presynaptic varicosities and terminal boutons containing clusters of vesicles opposed to postsynaptic densities that are formed either on the dendritic shaft or onto spinous structures that protrude from the shaft. These well-defined structures are separated by the synaptic cleft, which normally appears granular indicating proteinaceous composition. Within this chapter I have discussed the

molecular composition of synapses and how functionally diverse proteins interact to regulate activity-dependent neurotransmitter release and SV recycling.

Presynaptic structure and function is fundamentally the same for both excitatory and inhibitory synapses. In contrast, postsynaptic structure and function is vastly different at excitatory and inhibitory synapses. Excitatory postsynaptic sites are predominantly formed onto actin-rich spines that extend out of the dendritic shaft. Spines provide physical compartments to restrict the complex biochemical signaling that underscores synaptic plasticity. Glutamate binding with AMPA-Rs and NMDA-Rs induces cation influx, which depolarizes the postsynaptic membrane and triggers multiple signaling pathways including CaMKII activation. CaMKII subsequently phosphorylates PSD scaffolding proteins, which in turn regulates receptor function and trafficking, as well as actin dynamics. PSD composition, receptor function and actin dynamics are all critical regulators of spine morphology, function and strength. In contrast to excitatory spines, inhibitory postsynaptic sites form directly on the dendritic shaft. Inhibitory postsynaptic sites contain receptors that are selectively permeable to anions, which hyperpolarize the postsynaptic membrane. Membrane hyperpolarization effectively modulates the propagation of action potentials along the dendrite by altering the membrane potential.

The balance of excitatory and inhibitory synapses is critical to normal brain function and is tightly controlled by mechanisms that are currently poorly understood. Transynaptic complexes of NX-NL are the best-characterized candidate molecules for regulating the number of excitatory and inhibitory synapses. Importantly, studies reveal that activity regulates and coordinates the number and size of both excitatory and inhibitory PSDs in the mature brain, suggesting a homeostatic process balances excitation and inhibition. This is particularly significant given that synapses are highly dynamic structures that are formed throughout life (as discussed in the next section), and thus the correct balance of excitation and inhibition must be maintained.

1.3. Synapses are dynamic structures

The development of functional neural circuits requires the formation and elimination of axonal and dendritic arbors, and the assembly and disassembly of individual synapses. During early postnatal CNS development vast and excessive numbers of synapses form and functional neural circuits are subsequently sculpted out and refined by activity-dependent synaptic pruning (Eaton and Davis, 2003; Goda and Davis, 2003; Lichtman and Colman, 2000). In contrast, establishment of retinotopic maps in the *Xenopus* and zebrafish larvae tectum depends upon concurrent activity-dependent synapse assembly, stability and disassembly to incrementally direct neurite arbor structure (Meyer and Smith, 2006; Niell et al., 2004; Ruthazer et al., 2003; Ruthazer et al., 2006). In the mature brain, life-long experience and learning further modifies brain connectivity by strengthening and weakening selected synapses as well as stimulating the assembly of new synapses and the disassembly of existing ones (Alvarez and Sabatini, 2007; Holtmaat and Svoboda, 2009). However, in the normal brain functional neural circuits are

stable, and selected synapses are maintained for extended periods of time, possibly throughout the life of the organism (Grutzendler et al., 2002; Holtmaat et al., 2005; Trachtenberg et al., 2002). Therefore, mechanisms exist to confer extensive structural stability, whilst enabling a regulated turnover of synapses. From a cell biology perspective, understanding how synapses are assembled, disassembled and maintained is central to understanding how neural circuits are initially wired up and how synaptic and circuit plasticity in the adult brain is achieved. Within this section I shall describe the molecular and cellular mechanisms that regulate the dynamic processes of synapse assembly, disassembly and maintenance.

1.3.1.Synapse assembly

As described in the previous chapter, synapses are complex macromolecular structures composed of thousands of proteins. A fundamental question is therefore how do these molecules assemble? What are the triggers for their assembly into an organized synaptic terminal? How are synaptic components transported to the nascent synapse? And what are mechanisms that ensure they remain in place? In the past decade, our understandings of the cellular and molecular mechanisms that regulate synapse assembly have expanded considerably. Time-lapse imaging has revealed a temporal hierarchy of molecular assembly events, and genetic models and biochemical screening have elucidated many key proteins and signaling pathways that cooperate to coordinate the building of a synapse (McAllister, 2007; Scheiffele, 2003; Waites et al., 2005).

Here I will focus on our current understanding of the cellular and molecular processes of glutamatergic synaptogenesis within the CNS. Although some axo-axonic and dendro-dendritic synapses occur, the vast majority of CNS synapses form between axons and dendrites (Garner et al., 2006; Waites et al., 2005; Ziv and Garner, 2004), and for the purpose of this discussion I shall focus on axo-dendritic synapses.

Key cellular events of synaptogenesis

Synapse formation can be broadly divided into four phases, initial contact, induction, differentiation and maturation (Garner et al., 2006) (Figure 1.3). These divisions serve only as a discussion aid, as in reality the assembly of a synapse is a continual event.

- *Establishing axo-dendritic contact*

Once axons reach their target they must make dendritic contact to initiate synaptogenesis (Figure 1.3 panel A). However, it is unclear whether axons or dendrites initiate synapse assembly. Numerous time-lapse studies of neurons transfected with fluorescently tagged synaptic proteins VAMP2 and PSD95 reveal that navigating axons and dendrites leave transient nascent synapses in their wake, as they encounter one another, in an apparent stochastic and

promiscuous manner (Cline, 2001; Jontes et al., 2000; Meyer and Smith, 2006; Niell et al., 2004; Ruthazer et al., 2006). Both axons and dendrites extend highly motile filopodia from their growth cones and shafts, which exhibit protrusive behavior and appear to explore the environment in search of potential partners (Ahmari et al., 2000; Chang and De Camilli, 2001; Knott et al., 2006). Importantly, when contact is made pre and postsynaptic proteins are rapidly recruited to this site (Ahmari et al., 2000; Gerrow et al., 2006; Niell et al., 2004; Ruthazer et al., 2006; Sabo et al., 2006; Washbourne et al., 2002; Ziv and Smith, 1996), although many of these contacts have half-lives of just minutes (Holtmaat et al., 2005; Lohmann and Bonhoeffer, 2008; Niell et al., 2004; Okabe et al., 2001a; Sabo et al., 2006; Ziv and Smith, 1996).

The filopodial model for synaptogenesis was initially developed from in-vitro studies and purports that new spines grow towards axons and initiate axonal contact, which stimulates the formation of *de novo* boutons and the assembly of synapses (Ziv and Smith, 1996). Whilst *in-vivo* analyses supports the observation that dendritic filopodia display protrusive exploratory behavior, and contact nearby axons (5-10µm distance) to initiate filopodia stabilization and the formation of presynaptic boutons, these studies find that filopodia preferentially contact and make synapses with existing boutons, which suggests a presynaptic mechanism for attracting filopodia (Knott et al., 2006). Indeed, isolated presynaptic varicosities that cycle SVs can attract dendritic filopodia, presumably by releasing a trophic factor (Sabo et al., 2006). Whilst different axo-dendritic contact models have been described in terms of whether axons or dendrites drive synapse assembly, the evidence suggests that both axons and dendrites can initiate synaptogenesis, and it is possible that different mechanisms apply to different stages of CNS development.

Synaptic patterning of mature pyramidal neurons in the cortex is remarkably stereotyped, whereby synaptic inputs are arranged into distinct domains. For example, GABAergic inputs are predominantly located on the soma and axons, whereas Glutamatergic inputs are principally dendritic, with inputs from local neurons generally located on the proximal dendrites, and inputs from distant neurons usually situated on the distal apical tuft (Spruston, 2008). This raises the important question of whether specific synapses preferentially form at selective regions of the neurite, or whether initial synaptic patterning is random, as suggested by the stochastic nature of early differentiation described above. Evidence suggests that neurites may indeed contain defined, predisposed sites for synapse formation. Time-lapse studies of young cortical cultures transfected with VAMP2-GFP reveal that mobile SV clusters pause and cycle with the axonal membrane at defined sites (Sabo et al., 2006). Importantly, SV pausing occurs in the absence of dendrites neurons or glia, which suggests regulation by presynaptic mechanisms. Moreover, these sites differentiate into stable synaptic sites (Sabo et al., 2006). Further support of restricted synaptogenic sites is offered by evidence of stable postsynaptic PSD95/Neuroligin complexes within defined dendritic regions (Gerrow et al., 2006). These sites are reported to stimulate presynaptic differentiation (Gerrow et al., 2006). Taken together, these studies provide insights for the presence of determined sites where nascent synapses preferentially assemble.

- *Synaptic differentiation*

Synaptic differentiation is primarily triggered, regulated and coordinated through bi-directional signals across the synapse by secreted factors and cell membrane bound complexes (Craig et al., 2006; McAllister, 2007; Waites et al., 2005). *In-vitro* time-lapse analyses demonstrate axo-dendritic contact triggers a hierarchy of protein recruitment to nascent synaptic sites resulting in the assembly of active zones containing SV pools on the presynaptic side, and the PSD on the postsynaptic side (Ahmari et al., 2000; Friedman et al., 2000; Okabe et al., 2001a). Immunohistochemistry and Western blots from cultured neurons show that complexes of specific synaptic proteins are transported along axons and dendrites within vesicle structures. These studies suggest that AZs become functionally active before the assembly of postsynaptic scaffold proteins like PSD95, and glutamate receptors (Friedman et al., 2000; Okabe et al., 2001a). Presynaptic SV recycling is observed within ~15-30 minutes of initial contact, PSD95 starts to cluster ~45 minutes later, shortly followed by NMDA-Rs then AMPA-Rs (Friedman et al., 2000). Whilst discrepancies between the actual timing of these events have been reported (Washbourne et al., 2002), the general consensus is synaptic induction and initial differentiation occurs within 1-3 hours in cultured neurons (Figure 1.3 panels A and B).

Two categories of biochemically distinct presynaptic transport vesicles have been identified; Piccolo transport vesicles and SV protein transport vesicles (PVTs and SVTs respectively) (Ahmari et al., 2000; Zhai et al., 2001). PVTs originate from the trans-Golgi network (Dresbach et al., 2006) and transport AZ proteins such as Piccolo, Bassoon and N-Cadherin, as well as proteins required for SV exocytosis including Munc13 and Munc18, SNAP25 and syntaxin (Zhai et al., 2001). SVTs transport proteins associated with SVs and exo- and endocytosis, as well as calcium subunit α1a, SV2, synapsin1a and amphiphysan1 (Ahmari et al., 2000). PVTs and SVTs move anterogradely and retrogradely suggesting that multiple motor proteins are required for their transport (McAllister, 2007), yet the mechanisms that trigger and direct their transport to nascent sites are not yet understood.

Mobile clusters of postsynaptic proteins are also evident within dendrites prior to the formation of synapses in immature neuronal cultures. Time-lapse studies of immature hippocampal cultures (4-6 DIV) reveal that Neuroligin1-GFP (NL1) clusters are mobilized to active filopodia within minutes of axo-dendritic contact, which stimulates the recruitment of PSD95 (Barrow et al., 2009). NMDA-Rs and AMPA-Rs are trafficked independent of each other along microtubules (Gerrow et al., 2006; Washbourne et al., 2002; Washbourne et al., 2004) and exhibit considerably different rates of transit (Washbourne et al., 2002). In addition to unitary protein recruitment, preassembled complexes of PSD95, GKAP and Shank have been identified (Gerrow et al., 2006), suggesting that postsynaptic transport packets may be trafficked to and incorporated into nascent synaptic sites. PSD95 and NMDA-Rs is also recruited from local diffuse dendritic pools of proteins (Bresler et al., 2004). Furthermore, the presence of ribosomes and mRNAs within the dendrite indicates that postsynaptic proteins are translated locally and integrated into synaptic sites (Bourne and Harris, 2010; Kennedy and Ehlers, 2006).

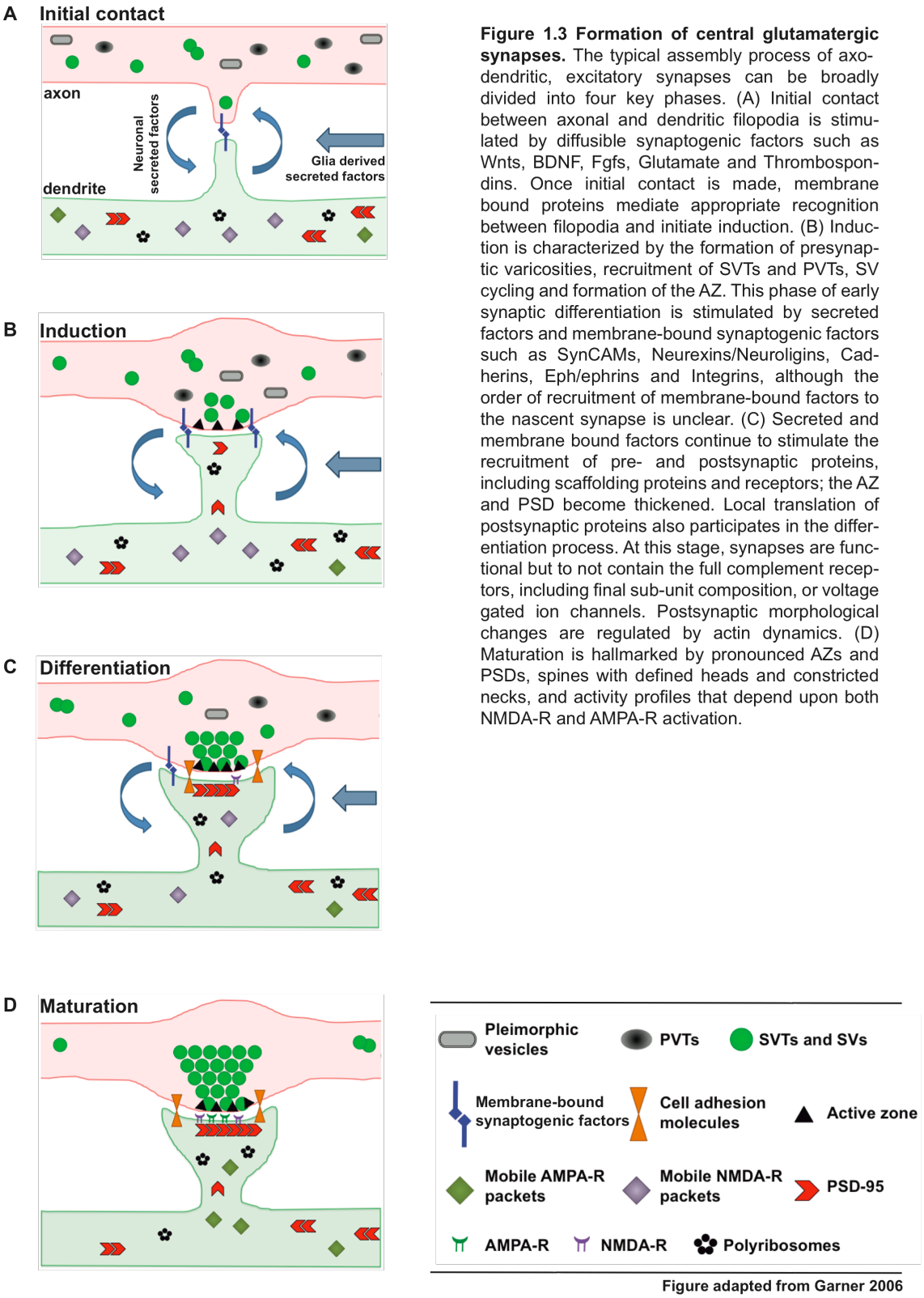


Figure adapted from Garner 2006

Figure 1.3 Formation of central glutamatergic synapses. The typical assembly process of axo-dendritic, excitatory synapses can be broadly divided into four key phases. (A) Initial contact between axonal and dendritic filopodia is stimulated by diffusible synaptogenic factors such as Wnts, BDNF, Fgfs, Glutamate and Thrombospondins. Once initial contact is made, membrane bound proteins mediate appropriate recognition between filopodia and initiate induction. (B) Induction is characterized by the formation of presynaptic varicosities, recruitment of SVTs and PVTs, SV cycling and formation of the AZ. This phase of early synaptic differentiation is stimulated by secreted factors and membrane-bound synaptogenic factors such as SynCAMs, Neurexins/Neuroligins, Cadherins, Eph/ephrins and Integrins, although the order of recruitment of membrane-bound factors to the nascent synapse is unclear. (C) Secreted and membrane bound factors continue to stimulate the recruitment of pre- and postsynaptic proteins, including scaffolding proteins and receptors; the AZ and PSD become thickened. Local translation of postsynaptic proteins also participates in the differentiation process. At this stage, synapses are functional but to not contain the full complement receptors, including final sub-unit composition, or voltage gated ion channels. Postsynaptic morphological changes are regulated by actin dynamics. (D) Maturation is hallmarked by pronounced AZs and PSDs, spines with defined heads and constricted necks, and activity profiles that depend upon both NMDA-R and AMPA-R activation.

- *Synapse Maturation*

The initial stages of synaptic differentiation occur within just a few hours after contact. However, synapse maturation is a protracted process that involves the final assembly of hundreds of proteins, significant morphological changes and the development of electrophysiological properties (Figure 1.3 panel D) (Knott et al., 2006; Nagerl et al., 2007; Zhang and Benson, 2001). Structurally, synapses continue to grow larger as pre- and postsynaptic proteins continue to accumulate (McAllister, 2007); presynaptic SVs continue to cluster proximal to the AZ and the SV population becomes more heterogeneous, containing less pleiomorphic vesicles and more spherical SVs (Ahmari et al., 2000). Postsynaptically, the PSD becomes more pronounced (Tada and Sheng, 2006; Yuste and Bonhoeffer, 2004), and young spines undergo dramatic structural changes forming constricted necks with defined "heads", which may then proceed to develop into mushroom or stubby spines (Knott et al., 2006; Yuste and Bonhoeffer, 2004; Ziv and Smith, 1996).

Actin is a key player in regulating synapse formation and maturity. Disruption of actin polymerization by latrunculinA significantly reduces the number of synaptic sites in immature cultured neurons. In contrast, mature neurons are significantly more resilient and the majority of synaptic sites persist after latrunculinA treatment (Zhang and Benson, 2001). This resilience to latrunculineA correlates with the progressive accumulation of synaptic scaffold proteins Bassoon and PSD95. Together with other studies (Allison et al., 2000), the evidence suggests synaptic molecules are initially tethered to immature synapses via the actin cytoskeleton through actin binding proteins. As the synapse matures, interactions with the intracellular matrix of scaffold proteins provide an additional stabilizing platform.

The progressive accumulation of scaffolding proteins such as PSD95, Shank and Homer confer synaptic functionality through functional domains that effectively recruit neurotransmitter receptors and signaling molecules to the PSD (El-Husseini et al., 2000; Kim and Sheng, 2004; Sala et al., 2001). The postnatal brain contains a significant proportion of electrically "silent" synapses, which contain NMDA-Rs but not AMPA-Rs (Isaac et al., 1995; Kerchner and Nicoll, 2008). For example, in the first few postnatal days nearly all Schaffer collateral-CA1 synapses are electrically silent, whereas by week 3 approximately 50% have acquired AMPA-Rs and are electrically active (Kerchner and Nicoll, 2008). The recruitment of AMPA-Rs to the PSD is an activity-dependent process and is a critical phase of synapse maturation. In addition to the type of Glutamate receptor present at the maturing synapse, NMDA-R subunit configuration is altered. In most brain regions, changes in postsynaptic NMDA-R subunit composition occur during the second and third postnatal week, whereby NR2B is gradually replaced with NR2A (Cull-Candy et al., 2001; Petralia et al., 2005; Sans et al., 2000). NMDA-R subunit composition significantly affects the timecourse of decay of NMDA-R mediated EPSCs; NR2A induces a

much quicker NMDA-R EPSC decay compared to NR2B, which is associated with experience-dependent synaptic plasticity (Cull-Candy et al., 2001).

Synapse maturation also involves significant changes to ion channel types and chloride transporter pumps. Different calcium channel types confer different conductance properties, which regulate neurotransmitter release activation (McCleskey, 1994). Electrophysiological recordings from cultured hippocampal neurons treated with specific calcium channel toxins reveal a progressive switch from presynaptic N-type voltage gated calcium channels (VGCC) to Q-type during weeks 1-3, whereby mature synapses predominantly express Q-type channels (Scholz and Miller, 1995). Importantly, Q-type channels are more effective that N-type channels at eliciting neurotransmitter release (Catterall and Few, 2008). As previously described, postsynaptic chloride transporter proteins radically modify synaptic transmission properties during synapse maturation by regulating the reversal potential for $GABA_AR$ mediated responses and switches from GABA mediated excitatory transmission to inhibition. Therefore, synapse maturation involves multiple changes in the recruitment and arrangement of ion channels and neurotransmitter receptor subtype, which together regulate electrical properties of synapses.

In contrast to the initial phases of synaptic differentiation, which are rapid and enable immediate neural responses to stimuli, the complex modifications required for synaptic maturity are protracted events that occur over many hours (Nagerl et al., 2007). The structural and functional changes that hallmark synapse maturation depend upon activity-dependent mechanisms and the temporal expression of critical receptor subunits, ion channels and transporters. As described, the initial phases of synaptogenesis are promiscuous events; it is therefore possible that the extended timecourse for maturation provides windows of opportunity for mechanisms to validate required synapses before they become fully mature, and arrest development of unwanted synapses.

Molecular Mechanisms of synapse assembly

The past decade has revealed a vast number of synaptogenic molecules, which are broadly categorized into secreted factors and membrane-bound signaling molecules. Motile axonal and dendritic filopodia are active prior to contact and “seek-out” partners within the immediate environment suggesting that these processes sense their environment (Chang and De Camilli, 2001; Jontes et al., 2000; Lohmann and Bonhoeffer, 2008; Ziv and Smith, 1996). This behavior is most probably mediated by secreted factors from either axons or dendrites or both. Indeed, a number of target-derived, secreted axon-guidance molecules are now understood to be important regulators of synaptogenesis (Shen and Cowan, 2010). Synaptic partners touch and retract from each other repeatedly before contact is stabilized and the vast majority of initial encounters do not stabilize (Knott et al., 2006; Lohmann and Bonhoeffer, 2008; Trachtenberg et al., 2002). Such promiscuous behavior suggests that mechanisms exist for appropriate recognition between potential partners/targets, which are most likely controlled by membrane-bound molecules. However, such a simple division of labor is not necessarily appropriate, as

described below, because many secreted factors also participate in subsequent stages of synaptogenesis and synapse maturation. Here I shall describe the most characterized molecular families that have been demonstrated to stimulate synaptogenesis.

- *Secreted Factors*

In the CNS, prior to axo-dendritic contact, preliminary proto-synaptic differentiation is observed, for example presynaptic SVs cycle at the membrane without postsynaptic apposition (Dai and Peng, 1996; Kraszewski et al., 1995; Krueger et al., 2003; Matteoli et al., 1992). Furthermore, postsynaptic clusters of membrane associated NMDA receptors (Washbourne et al., 2004) and stable scaffold complexes aggregate without presynaptic contact (Gerrow et al., 2006). Pre-contact synaptic differentiation suggests that neurites either intrinsically form clusters of synaptic proteins, or, mechanisms exist that enable neurites to sense ambient factors, such as secreted proteins, which stimulate synaptic differentiation. Within this section, I will focus my discussion on the key secreted molecular players that are involved in CNS synapse development and maturation, which include BDNF, FGFs, Wnts, glutamate, and the glial-derived factors cholesterol and thrombospondin.

BDNF

Brain derived neurotrophic factor (BDNF) is a prominent and well-characterized secreted synaptogenic factor in the CNS, that also contributes to neuronal survival and neurite branching (Lessmann et al., 2003; McAllister et al., 1999; Poo, 2001). BDNF is secreted in an activity dependent manner (Yoshii and Constantine-Paton, 2007) and its pro-synaptic activity was demonstrated in the mid 1990s from studies of ocular dominance column (ODC) formation in the cat visual cortex (Cabelli et al., 1995). ODC formation depends upon a critical balance between synapse formation and elimination in an activity dependent manner; local infusion of BDNF into the primary visual cortex significantly impairs ODC development by shifting the balance towards excess synapse formation (Cabelli et al., 1995). Furthermore, studies of transgenic mice demonstrate that BDNF overexpression alters the critical period for ocular dominance plasticity by accelerating synapse and circuit maturation (Hanover et al., 1999; Huang et al., 1999).

BDNF signaling, through its tyrosine kinase receptor TrkB, is implicated in both pre and postsynaptic mechanisms during synapse formation (McAllister et al., 1999; Poo, 2001). TrkB receptors are localized to both axonal and dendritic filopodia, (Gomes et al., 2006) and the temporal expression of synaptic surface TrkB is consistent with a role for BDNF as an early mediator of synaptogenesis (Gomes et al., 2006). Electronmicrographs taken from mice with a conditional deletion of presynaptic TrkB reveal a reduction in the number of axonal varicosities (Danzer et al., 2008; Luikart et al., 2005). Furthermore, the number of SVs clustered around AZs in persisting synapses in TrkB knockout mice are significantly reduced (Martinez et al., 1998). Deficits in TrkB signaling also result in decreased expression of the SV associated

proteins Synapsin1 and Synaptophysin, as well as the SNARE proteins Synaptotagmin and SNAP25 (Martinez et al., 1998). A number of studies have demonstrated that BDNF facilitates neurotransmitter release (Lessmann et al., 2003; Poo, 2001) possibly by increasing cytosolic calcium (Berninger et al., 1993; Stoop and Poo, 1996) and/or regulating SNARE protein expression (Martinez et al., 1998).

Postsynaptically, immunocytochemistry and fluorescent recovery after photobleaching (FRAP) analyses reveal that bath application of BDNF increases co-localization between TrkB and PSD95 and facilitates PSD95 trafficking to synaptic sites (Yoshii and Constantine-Paton, 2007). Furthermore, biochemical analyses show TrkB and PSD95 binding depends on kinase activity of Akt through P13K signaling, which are key molecular intermediates in the BDNF pathway (Yoshii and Constantine-Paton, 2007). Blockade of presynaptic neurotransmitter release with specific calcium channel blockers does not affect BDNF induced PSD95-GFP FRAP, which discounts the possibility that presynaptic neurotransmitter release contributes to these results (Yoshii and Constantine-Paton, 2007). Conditional knock out mice that selectively ablate postsynaptic TrkB postnatally exhibit a decrease in the frequency of mEPSCs, yet no change in the amplitude of mEPSCs is observed (Luikart et al., 2005). However, the amplitude of evoked EPSCs is significantly reduced in TrkB knockout cells compared to control cells (Luikart et al., 2005). These data suggest that Trk knockout cells have fewer functional excitatory postsynaptic sites and the synapses that are present are postsynaptically unresponsive to stimulus. Taken together, these studies demonstrate a role for BDNF signaling both pre- and postsynaptically in the formation of synapses.

Bath application of BDNF increases the frequency and amplitude of evoked and miniature IPSCs *in-vitro*, (Aguado et al., 2003). The number of functional excitatory synapses is also elevated by TrkB overexpression (Aguado et al., 2003). Postsynaptic overexpression of BDNF-GFP in isolated cultured neurons from BDNF null mice preferentially stimulates the formation of excitatory synapses and suppresses the formation of inhibitory synapses, therefore increasing the excitatory/inhibitory ratio (Singh et al., 2006). This study suggests that BDNF signaling may be involved in regulating the balance of excitation and inhibition. However, *in-vivo* overexpression of BDNF increases the number of inhibitory synapses and accelerates GABAergic circuit formation (Aguado et al., 2003; Cabelli et al., 1995) and increases expression of the K^+Cl^- transporter KCC2 (Aguado et al., 2003). Whether these differences reflect different neuronal behaviors *in-vivo* versus *in-vitro,* or properties of BDNF function that have yet to be characterized, has yet to be understood.

FGFs

Fibroblast growth factors (FGFs) are a conserved family of heparin-binding growth factors that are involved in multiple aspects of embryonic and postnatal development including synaptic differentiation and neurite branching (Bottcher and Niehrs, 2005; Itoh and Ornitz, 2008; Mason, 2007; Reuss and von Bohlen und Halbach, 2003). In mammals, 22 FGF family members have been identified; in contrast, only 4 FGF receptor (FGF-R) genes are known (Itoh and Ornitz,

2008). The extracellular domain of FGF-Rs contains alternative splice sites that generate isoforms, which have been found to confer ligand specificity (Zhang et al., 2006). Intracellular, FGF-Rs contain a tyrosine kinase domain with the capacity for multiple downstream signaling cascades (Zhang et al., 2006).

In cultured spinal cord neurons, localized FGF2 application by coated microspheres or microinjection stimulates presynaptic SV clustering in a calcium dependent manner (Dai and Peng, 1995). In hippocampal cultures, FGF2 mediated presynaptic differentiation is associated with SV cycling sites and postsynaptic clustering of PSD95 and GluR1 clusters (Li et al., 2002). Whilst these data suggest that FGF2 can stimulate the assembly of functional synapses, in the study by Li and colleagues FGF was applied chronically over a period of 6 days. Therefore it is unclear whether FGF2 acts directly on the postsynaptic side to stimulate differentiation, or whether presynaptic assembly and function mediate such effects.

In-vivo and *in-vitro* studies reveal that FGF7 and FGF22 signaling in the cerebellum and hippocampus regulates SV clustering (Terauchi et al.; Umemori et al., 2004). Conditional FGFR-2 mutant mice, which are deficient in FGF22 signaling, display reduced Synapsin1 and Synaptophysin clustering (Umemori et al., 2004). FGF7 and FGF22 deficient mice display similar defects in presynaptic differentiation; analysis of these mice reveals that the active zone, as detected by Bassoon clustering, remains unaffected suggesting that FGF7 and FGF22 explicitly regulate SV clustering without affecting active zone assembly (Terauchi et al.). Importantly, this study shows clustering of postsynaptic scaffold proteins PSD95 and Gephyrin are unaffected, which suggests FGF7 and FGF22 signaling specifically regulates presynaptic differentiation. Furthermore, FGF7 and FGF22 deficient mice specifically exhibit defects in the clustering of SVs that contain vGat and vGlut respectively, suggesting that specific FGF isoforms selectively stimulate the assembly of inhibitory or excitatory synapses through presynaptic mechanisms. Therefore, FGF signaling is an important synaptogenic factor that regulates SV clustering, and is involved in specifying inhibitory and excitatory synaptic transmission. Further analyses of the many FGF family members, their cognate receptors and the downstream signaling pathways will undoubtedly reveal further roles for FGF signaling at the synapse.

Wnts

Wnts are a conserved family of secreted protoglycans that are functionally diverse and required for many critical stages of embryonic development and CNS wiring (Budnik and Salinas, 2011; Ciani and Salinas, 2005; Freese et al., 2010; Salinas and Zou, 2008). The diversity of function is achieved through multiple Wnt ligands and their equally diverse cognate receptors together with co-receptors and differential temporal expression patterns (Angers and Moon, 2009; Chien et al., 2009; Kikuchi et al., 2007). The data presented within this thesis expands upon our current knowledge of Wnt signaling at the synapse and explores the underlying mechanisms. Accordingly, an entire section is dedicated to a discussion of Wnt signaling and its role at the synapse, and will not be discussed further here.

Glutamate

Glutamate is the principle excitatory neurotransmitter in the CNS and is transferred from the cytoplasm into SVs via vesicular Glutamate transporters (vGlut) embedded within the SV plasma membrane (Takamori, 2006). The specificity of vGlut as a marker for Glutamate makes this protein a reliable marker for detecting glutamatergic SVs (Takamori, 2006). Detection of vGlut within presynaptic SV cycling clusters that are not opposed to postsynaptic sites suggests that glutamate is released presynaptically prior to axo-dendritic contact (Sabo et al., 2006). Importantly, motile dendritic filopodia are selectively attracted to vGlut positive clusters (Sabo et al., 2006). Increased dendritic filopodia motility and outgrowth is regulated by NMDA-R activation (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Toni et al., 1999). However, once axo-dendritic contact and synapses start to differentiate glutamatergic transmission stabilizes filopodia through AMPA-R and Kainate receptor activation (Chang and De Camilli, 2001; Fischer et al., 2000; Tashiro et al., 2003). Together, these studies provide support for a two-step model of Glutamate regulating synapse formation; initially by stimulating filopodia motility, which would help filopodia find a synaptic partner, then an inhibitory effect once contact is made, which acts to stabilize the nascent synapse (Fischer et al., 2000; Tashiro et al., 2003).

Glutamatergic transmission regulates neurite filopodia motility by modulating actin dynamics (Chang and De Camilli, 2001; Tashiro et al., 2003; Wong and Wong, 2001; Zheng et al., 1996). Neural stimulation regulates the translocation of the actin binding proteins (ABPs) such as Profilin (Ackermann and Matus, 2003) and Cortactin (Hering and Sheng, 2003), and structural proteins that interact with the cytoskeleton such as N-cadherin and β-catenin (Bozdagi et al., 2000; Tai et al., 2007) in and out of synapses. The association of actin with ABPs and structural proteins stabilize the actin cytoskeleton, which inhibits filopodia motility (Dillon and Goda, 2005). The mechanisms of Glutamate receptor regulation of actin dynamics has already been discussed (see Chapters 1.2.2.) and is further addressed in Chapter 1.3.3 with reference to synaptic maintenance. Consequently, I will not further the discussion of activity-dependent actin dynamics at this point, other that to underscore its role in modulating filopodia development and therefore synapse assembly.

There is a compelling body of work that proposes activity, through glutamate release, regulates synaptogenesis (Holtmaat and Svoboda, 2009; Hua and Smith, 2004). However, studies also demonstrate that glutamate signaling is dispensable for synapse formation (Harms and Craig, 2005; Lohmann and Bonhoeffer, 2008). Synapses can form in the absence of glutamate release, as revealed by analysis of mice deficient in Munc18, which are defective in both spontaneous and evoked neurotransmitter release (Rosenmund et al., 2002; Verhage et al., 2000). Whilst the gross architecture of the brain in these mice is normal there is a significant decrease in the total number of cortical synapses, and the synapses that do develop fail to mature (Bouwman et al., 2004). It is unclear whether the decrease in synapse number reflects initial synaptogenic failures, or whether the normal complement of synapses successfully

assemble then consequently disassemble due to defects in activity dependent maturation. Therefore, secreted glutamate may play a subtler role in establishing axo-dendritic contact that is subject to redundancy.

Glia secreted synaptogenic factors

Glial cells are intimately associated with neurons and are an important constituent of the neuropil supporting neural development and function (Eroglu and Barres, 2010; Fellin, 2009; Perea et al., 2009). At a passive level, glia cells regulate ion homeostasis and neurotransmitter concentrations in the synaptic cleft by providing physical constraints against diffusion. Glia are also active partners at the synapse and participate in neural function through secretion of factors that regulate synapse assembly and plasticity (Allen and Barres, 2005; Ullian et al., 2004). Neurons cultured in the absence of glia or glia-conditioned medium contain fewer synapses, and those that are present display reduced frequency and amplitude in both spontaneous and evoked synaptic transmission (Nagler et al., 2001; Pfrieger and Barres, 1997). Accordingly, addition of glial-conditioned medium increases the number of functional synaptic sites (Nagler et al., 2001; Pfrieger and Barres, 1997; Ullian et al., 2004; Ullian et al., 2001).

Biochemical fractionation studies of glial-conditioned medium have identified apolipoproteinE complexed with cholesterol as a glia-derived synaptogenic factor (Mauch et al., 2001). Long-term exposure of exogenous cholesterol in cultured retinal ganglion cells significantly increases the number of differentiated pre- and postsynaptic sites and the frequency of spontaneous EPSCs (Goritz et al., 2005; Mauch et al., 2001). Whilst cholesterol also stimulates dendritic outgrowth and differentiation (Goritz et al., 2005), the elevated synaptic number is independent of any increase in neurite length (Mauch et al., 2001). Interestingly, the synaptogenic effects of cholesterol require 24-48 hours exposure, which suggests multi-stepped mechanisms that may be transcriptionally mediated (Mauch et al., 2001). *In-vitro,* neurons are capable of secreting sufficient cholesterol to develop and mature, however, the vast number of synapses generated *in-vivo* requires additional cholesterol, which is generated by glial cells (Mauch et al., 2001). This indicates that cholesterol acts *in-vivo* as a synapse-limiting factor in the CNS; only once sufficient glia cells have been generated to support developing neurons, is sufficient cholesterol produced to stimulate/enhance synaptogenesis (Mauch et al., 2001).

Thrombospondins (TSPs) are secreted from astrocytes and bind with multiple membrane receptors and ECM proteins to mediate cell-cell and cell-matrix interactions (Adams, 2001; Bornstein, 2001; Christopherson et al., 2005). There are five TSP family members, TSP1-5; TSP1 and TSP2 are highly related and share common functional motifs (Adams, 2001). Importantly, immunodepletion studies demonstrate that both TSP1 and TSP2 are required in astrocyte-conditioned medium to stimulate the formation of synapses that are ultrastructurally normal and contain the same immunohistochemical profiles as proteins such as Synaptotagmin and PSD95 (Christopherson et al., 2005). The pattern of synaptogenic activity of TSP1 and TSP2 correlates with endogenous expression levels, which are enriched postnatally by P8, but reduced by P21. Importantly, TSP1/2 double null mice have 40% fewer synapses at P8, yet the

number of cells and gross morphology of cortical structures and layers remains unaltered. These data support the hypothesis that TPS1 and TSP2 act transiently and specifically to promote synaptogenesis in the immature brain (Christopherson et al., 2005). However, TSP stimulated synaptogenesis requires chronic exposure to TSP1/2 (6 days); the authors discuss the extended time period required to observe TSP effects may reflect TSP induced expression of proteins required for synaptogenesis. It is also possible that TSPs participate in stabilizing nascent synapses, which is consistent with their characterized adhesive properties (Adams, 2001). Indeed, TSPs interact with synaptic transmembrane proteins such as integrins (Venstrom and Reichardt, 1993) and neuroligins, which place them well for cooperating with adhesion and membrane proteins. Therefore, whilst TSPs are important secreted synaptogenic factors, their mechanisms remain poorly understood at this time.

- *Membrane-bound signaling molecules*

Many axo-dendritic contacts are transient, lasting just seconds or minutes (Holtmaat et al., 2005; Lohmann and Bonhoeffer, 2008; Niell et al., 2004; Okabe et al., 2001a; Sabo et al., 2006; Ziv and Smith, 1996), which suggests that a process of recognition and selection occurs during initial contact. Local dendritic calcium transients have been reported to occur with 10-40 seconds of contact in a glutamate independent manner indicating that contact-induced calcium transients are independent of neuronal activity, and are likely to be triggered by signaling molecules (Lohmann and Bonhoeffer, 2008). Importantly, these calcium transients were only observed once axo-dendritic contact had been made, which suggests that membrane bound signaling, rather that secreted factors may be regulating this phenomenon. In addition to recognition properties, membrane bound signaling molecules play essential roles in synaptic differentiation, functional maturation and maintenance.

SynCAMs

SynCAMs (synaptic cell adhesion molecules) are transynaptic adhesion molecules that are part of the immunoglobulin superfamily (Biederer, 2006). SynCAM expression strongly correlates with the peak of synaptogenesis and only minimal levels are detected in the adult brain, suggesting SynCAMs primarily regulate synaptogenesis rather than synapse function (Fogel et al., 2007). The four SynCAM family members (SynCAM1-4) identified cooperate in synapse assembly (Biederer, 2006). SynCAM1 is expressed throughout the postnatal hippocampus (P15) and SynCAMs-2-4 display differential, but overlapping, spatial expression patterns with one another (Biederer, 2006; Thomas et al., 2008). SynCAMs1-4 are localized within pre and postsynaptic compartments where they form Ca^{++} independent homophyllic and heterophyllic interactions across the synaptic cleft via immunoglobulin domains that are regulated by glycosylation (Biederer et al., 2002; Fogel et al., 2007). Heterophilic pairs preferentially form between SynCAM-1 and -2, SynCAM-2 and -4 and SynCAM-3 and -4 (Fogel et al., 2007; Thomas et al., 2008). SynCAMs localize to both excitatory and inhibitory synapses, however, SynCAMs1 and -2 preferentially localize to excitatory synapses suggesting that SynCAM

isoforms, or specific heterophillc pairings, may play a role in the balance of excitatory and inhibitory synapses (Biederer, 2006; Sara et al., 2005) possibly by cooperating with specific Neuroligin isoforms (Sara et al., 2005).

Postsynaptic overexpression of SynCAM1 significantly increases the number of presynaptic SV cycling sites and increases excitatory mEPSC frequency but not the amplitude, indicating that postsynaptic SynCAM regulates presynaptic differentiation and function (Biederer et al., 2002; Fogel et al., 2007). However, exogenous Glutamate triggers strong EPSC amplitudes in non-neuronal cells (293 cells) co-transfected with SynCAM1 and GluR2, compared to 293 cells expressing only SynCAM1 or GluR2 (Biederer et al., 2002). This study implies that 293 cells expressing both SynCAM1 and GluR2 display postsynaptic glutamatergic responses that resemble normal brain synapses and strongly suggests that SynCAM1 participates in functional postsynaptic differentiation (Biederer et al., 2002; Sara et al., 2005). SynCAMs contain a cytoplasmic PDZ domain that binds with CASK, but not PSD95, which is the likely mechanism for SynCAM mediated presynaptic function (Biederer et al., 2002). The postsynaptic mechanism for SynCAM mediated synaptic differentiation has yet to be elucidated. Whilst the study by Biederer (2002) finds no SynCAM/PSD95 interactions, these experiments were conducted before identification of the different SynCAM isoforms, and therefore characterization of specific isoform interactions remain incomplete. Furthermore, it has been postulated that due to the similar cytosolic protein interaction domains shared by SynCAMs and Neurexin/Neuroligins, postsynaptic SynCAM may well operate through comparable mechanisms, which involve PDZ interactions with PSD95 (Fogel et al., 2007).

Neurexin/neuroligin complexes

The role of Neuroligin-Neurexin (NX-NL) complexes as synaptogenic factors has been intensively studied (Chih et al., 2005; Dean et al., 2003; Graf et al., 2004; Heine et al., 2008; Lee et al., 2010; Levinson et al., 2005; Nam and Chen, 2005; Prange et al., 2004; Scheiffele, 2003; Scheiffele et al., 2000). Transynaptic NX-NL complexes are rapid and potent inducers of pre and postsynaptic differentiation *in-vitro*. Postsynaptic NLs bind with presynaptic Neurexins at specific extracellular sites that are regulated by alternative splicing (Boucard et al., 2005; Ichtchenko et al., 1996; Lee et al., 2010). Presynaptically, NX-NL binding induces SV clustering (Scheiffele et al., 2000) through interactions with CASK, Munc-18 and Synaptotagmin (Biederer and Sudhof, 2000; Hata et al., 1996; Hata et al., 1993a). Postsynaptically, NX-NL binding stimulates synaptic differentiation through intracellular interactions with postsynaptic scaffold proteins PSD95 and Gephyrin (Graf et al., 2004; Levinson et al., 2005), and NMDA-Rs (Barrow et al., 2009). Importantly, NX-NL induced synapse assembly generates functionally active synapses (Chih et al., 2005; Prange et al., 2004). As previously described (Chapter 1.2.3), NX-NL transynaptic complexes specify excitatory or inhibitory transmission properties (Craig et al., 2006; Craig and Kang, 2007; Dean and Dresbach, 2006; Lise and El-Husseini, 2006). Postsynaptic NL-1, -3 and -4 localize to excitatory synapses, whereas NL2 preferentially localizes to inhibitory synapses (Graf et al., 2004; Prange et al., 2004; Song et al., 1999;

Varoqueaux et al., 2004). The preferential stabilization of NL isoforms to specific synapses is regulated through functional intracellular C-terminal domains, including PDZ motifs, which recruit and bind to scaffolding proteins and signaling molecules that in turn specify synapse identity (Levinson et al., 2010). Neurexin-Neuroligin isoforms complexes therefore provide a platform for coordinated induction of pre and postsynaptic synaptic differentiation.

Time-lapse analyses of cultured cortical neurons have recently identified two pools of surface NL1, diffuse and clustered (Barrow et al., 2009). Axo-dendritic contact stimulates aggregation of NL1 from a diffuse pool, where it translocates to the point of contact within minutes of initial contact (Barrow et al., 2009). NL1 clusters move in and out of active filopodia, and 26% of all filopodia contain NL1 clusters at the tip, which is the filopodial point of contact (Barrow et al., 2009). Many NL1 clusters are directly associated with NMDA-Rs, and evidence suggests NL1 is capable of recruiting both NMDA-Rs and PSD95 to nascent synapses (Barrow et al., 2009).

The role for NX-NL complexes *in-vivo* synaptogenesis is less clear. Surprisingly, mice mutant in NL1, -2 and -3 develop the normal complement of synapses and the gross cytoarcitecture of the brain remains indistinguishable from heterozygous littermates (Varoqueaux et al., 2006). However, Western Blot analyses from whole brain lysates reveal an intriguing profile of protein expression in the triple KOs; whilst the expression of AZ constituents, PSD95 and AMPA-Rs remain unaffected, there is a significant reduction in SV markers, SNARE proteins and NMDA-Rs (Varoqueaux et al., 2006). Unfortunately, the authors did not report on whether any changes in synaptic protein levels were observed at synaptic sites. Electrophysiological recordings from cultured cortical neurons form newborn triple KO mice reveal no significant changes in the frequency and amplitude of AMPA-R and NMDA-R mediated mEPSCs compared to heterozygous littermates (Varoqueaux et al., 2006). However, the triple KO mice die shortly after birth from respiratory failure due to substantial deficits in network activity within the respiratory regions of the brainstem, even though the number of synapses is not affected. These regions are normally functionally mature by birth, which suggests that NL1, -2, and -3 are not required for the initial formation of synapses *in-vivo*, but are required for synapse maturation and function in mature synapses.

Integrins

Integrins are conserved heterodimeric transmembrane proteins that act as cell surface receptors. Isoforms that are expressed in the developing brain mediate heterophyllic interactions between adjacent cells or the extracellular matrix and cells (Wiesner et al., 2005), and stimulate calcium signaling (Coppolino et al., 1997). These roles could implicate integrin signaling during the initial contact phase and provide a mechanism for observed contact-dependent calcium transients (Lohmann and Bonhoeffer, 2008). Importantly, Integrins regulate actin dynamics via RhoGTPases and lipid kinases (Wiesner et al., 2005), which regulate filopodia behavior and/or spine maturation. However, a role for Integrins during axo-dendritic contacts or initial induction of synaptic differentiation has yet to be demonstrated.

Studies in cultured neurons suggest that Integrin activity regulates functional synaptic maturation as blockade of β-3 Integrin signaling, via functional antibodies, alters the probability of glutamate release and prevents the activity-dependent switch of NMDA-R subunits that normally correlates with postsynaptic maturation (Chavis and Westbrook, 2001). By using a spectrum of inhibitors and functional analyses, the authors proposed a model whereby intracellular domains of postsynaptic integrins interact with tyrosine kinases to modulate NMDA-R subunit composition, and the cytoplasmic domains interact with an unknown presynaptic ligand to alter AZ size to affect release probability (Chavis and Westbrook, 2001). Functional analyses from conditional genetic deletion studies, using cre-recombinase lines, reveal that β-1 integrin regulates the number of docked SVs (Huang et al., 2006). These data support a role for integrin activity and presynaptic maturation and function. The authors (Huang et al., 2006) speculate that presynaptic β-1 integrin may be operating through talin, which binds to both the actin cytoskeleton and phosphatidylinositol-4,5-biphosphate (PIP_2). Importantly, PIP_2 has been shown to play a critical role in clathrin-mediated SV endocytosis and therefore directly impacts on SV recycling (Di Paolo et al., 2004). Intriguingly, talin is central to integrin function during focal complex formations and the turnover of focal adhesion points, through actin interactions (Wiesner et al., 2005). Talin is enriched at synapses where it regulates actin dynamics in the periactive zone (Morgan et al., 2004). However a role for talin/integrin function has not been reported in the initial formation of synaptic contact. Whilst Integrin function, in terms of regulating filopodia dynamics, cell adhesion and stimulating calcium signaling, places them well as mediators of synaptogenesis, their precise role during synapse assembly has yet to be fully elucidated.

Cadherins

The adhesion role of catenin-cadherin complexes is well characterized in epithelial cells, however their role at central synapses is still being unraveled. Cadherin-catenin transynaptic complexes are important regulators of synapse formation, stability and plasticity through both adhesion and signaling mechanisms. The extracellular domains of classical cadherins interact to form homo- and heterophyllic dimers in *cis* and *trans* (Arikkath and Reichardt, 2008). The cytoplasmic domains form complexes with multiple catenins (α-, β- and δ-catenin), which bind to a range of receptors, scaffold proteins, kinases, phosphotases and the actin cytoskeleton on both sides of the synapse (Arikkath and Reichardt, 2008; Yagi and Takeichi, 2000). Cadherin-catenin complexes therefore link transynaptic adhesion with diverse intracellular signaling cascades.

Nine different cadherins have been identified in the mammalian brain. In-situ hybridization studies show distinct temporal and spatial expression patterns, however, there is considerable overlap between multiple cadherins (Bekirov et al., 2002), which has impeded our understanding of how specific cadherins contribute to synapse assembly and function. N-cadherin is the most characterized cadherin in the brain; it is present at nascent synapses, and with development becomes restricted to the AZ. In mature synapses N-cadherin is localized to

peri-AZ regions and preferentially maintained at excitatory synapses (Arikkath and Reichardt, 2008). A number of overexpression and loss of function studies demonstrate that N-cadherin and β-cateinin complexes mediate both pre- and postsynaptic morphogenesis and function (Okuda et al., 2007; Saglietti et al., 2007; Stan et al., 2010; Togashi et al., 2002). Interactions between N-cadherins and β-catenins are stabilized by the cytoplasmic tyrosine kinase, Fer. Fer activity has been shown to be an important regulator of presynaptic differentiation (Lee et al., 2008b). Here, loss of Fer activity by shRNA resulted in fewer synaptophysin-GFP clusters and dispersed SV clusters, which was associated with reduced SV cycling and reduced EPSPs. Furthermore, spine morphogenesis was adversely affected (Lee et al., 2008b). These effects were mediated by Fer regulation of presynaptic β-catenin phosphorylation (Lee et al., 2008b). Based on these data, the authors suggest that Fer regulation of β-catenin regulates synaptic induction by localizing SVs and AZ components to nascent synapses.

Two recent studies posit that N-cadherin and Neuroligin1 (NL1) operate in concert, through a common signaling pathway, to regulate synapse assembly (Aiga et al., 2011; Stan et al., 2010). Importantly, these studies also reveal that N-cadherin is present at nascent synaptic sites before NL1, and that N-cadherin is an important molecular trigger to stimulate NL1 clustering, through interactions with the scaffolding protein S-SCAM (Stan et al., 2010). This suggests that N-cadherin is a very early membrane-bound inducer of synaptic differentiation.

Eph/ephrins

Eph/ephrins are transynaptic complexes that mediate contact-dependent, bidirectional signaling. Fourteen mammalian Eph receptor tyrosine kinases have been identified; nine EphAs and five EphBs (Klein, 2009). EphA receptors characteristically bind with A-type ephrins (ephrinA1-5) and EphB receptors with B-type ephrins (ephrinB1-3) (Klein, 2009). Ephrin ligands are typically localized to the presynaptic membrane, either through GPI linkage or a transmembrane domain with a cytoplasmic PDZ binding motif. Eph tyrosine kinase receptors are predominantly postsynaptic and in addition to their intracellular kinase domain, also contain PDZ binding motifs (Klein, 2009). Classical Eph/ephrin signaling is described as “forward” signaling, whereby the direction of signaling is from the presynaptic terminal to the postsynaptic terminal (Klein, 2009). However, Eph/ephrins have a unique property, Eph receptors can also act as ligands, and ephrin ligands can also act as receptors, such that presynaptic ephrins signal to their host cell; this is referred to as “reverse” signaling (Klein, 2009). For reverse signaling to occur, GPI linked ephrins associate with a transmembrane signaling partner, or the ephrin short cytoplasmic tail is modified by tyrosine or serine phosphorylation, which recruits signaling molecules (Klein, 2009).

EphB1-3 knockout mice show that forward signaling at the synapse is required for spine morphogenesis and postsynaptic differentiation (Henkemeyer et al., 2003). These mice exhibit fewer spines, reduced NMDA-R and AMPA-R clusters and PSD95 GFP is localized to dendritic shafts. Importantly, re-expression of EphB2 postnatally, rescues the spine defects and PSD95 GFP localization to spinous protrusions (Henkemeyer et al., 2003; Kayser et al., 2006). Forward

signaling through EphB receptors affects NMDA-R recruitment and function through direct interactions (Dalva et al., 2000), and actin dynamics via activation of RhoGTPases and the serine-threonine kinase PAK (Klein, 2009). Together these EphB forward signaling cascades cooperate to mediate postsynaptic differentiation (Klein, 2009).

Gain-of-function studies in the *Xenopus* retinotectal model, demonstrate that reverse signaling regulates presynaptic differentiation and function (Lim et al., 2008). Activation of presynaptic ephrinB1, through EphB2 fusion proteins, increases the number of synaptic sites and significantly enhances presynaptic glutamate release *in-vivo* (Lim et al., 2008). Expression of a truncated EphB2 isoform in co-culture studies suggests that the extracellular globular domain of EphB2 is sufficient to trigger axonal SV clustering through corresponding Ephrin interactions at contact points, however the presynaptic mechanisms remain unknown (Kayser et al., 2006). Further research is also required to understand when Eph/Ephrins are recruited to initial synaptic contact sites and how they cooperate with other classes of CAMs.

Summary of synapse assembly

The assembly of a synapse is an orchestrated collaboration between different molecular mechanisms. Secreted synaptogenic factors are released from axons, dendrites and glia to stimulate axo-dendritic contact and initiate synapse induction. Motile axonal and dendritic filopodia seek out potential partners and once contact is made, membrane bound synaptogenic proteins cooperate in the complex process of synaptic differentiation. The sequential recruitment of mobile pre and postsynaptic precursor molecules, scaffolding proteins and signaling molecules, each with different functional domains endows the nascent synapse with vast binding and signaling capacity. Together with developmentally regulated changes in receptor subunit composition, ion channels and transporters, synapses eventually mature and become physiologically active.

Whilst the amassed body of research has elucidated many key cellular mechanisms and molecules that together regulate synaptogenesis, significant unresolved issues remain. For example the role of activity; whether filopodia formation is constitutive or induced by secreted factors; whether axons or dendrites initiate contact or whether they both regulate assembly; the order of signaling events and how they collaborate with each other; the precise hierarchy of protein recruitment to nascent sites; and many of the fine mechanistic details of receptor signaling remain poorly understood. Indeed, will still don't really understand why many synaptic contacts are transient and what ultimately triggers the assembly of synapses or the initial triggers that determine the identity of a synapse in terms of transmission properties. As described above, there are a number of studies with different outcomes; such discrepancies are likely to reflect different experimental models, in particular different types of neurons and different stages of development. It is very likely that stages of circuit development employ different mechanisms of synapse assembly and indeed, different processes may occur in

parallel, for example pre and postsynaptic differentiation may be parallel events (Garner et al., 2006).

1.3.2.Synapse disassembly

Synapse disassembly is an effective mechanism for modulating connectivity between neurons. In the primate brain, all cortical regions undergo a simultaneous surge of synaptogenesis from approximately two months prior to birth up until 3-4 months postnatally; this intense expansion of synapse number is subsequently followed by a period of synapse elimination over the following 1-3 years (Rakic et al., 1986). Similar developmental patterns of exuberant synapse assembly and subsequent disassembly are observed in all vertebrates, albeit over different timescales (Lichtman and Colman, 2000). Tightly regulated synapse shrinkage and disassembly are also associated with functional plasticity in the adult brain (Bastrikova et al., 2008; Holtmaat and Svoboda, 2009; Zhou et al., 2004) and aberrant disassembly during development and in adulthood underpins many neurological disorders (Selkoe, 2002). This chapter is divided into discussions of developmentally regulated synapse disassembly during the early postnatal period; the underlying mechanisms that regulate selective synapse disassembly; synapse disassembly in the young and adult brain; and the role of aberrant synapse loss as a trigger for neurodegeneration.

Synapse disassembly during early postnatal development

Stereotyped developmental programs of synapse assembly and disassembly occur in both the central and peripheral nervous system during postnatal development. The vast excess of synapses that are formed during early development generates diffuse and redundant connections; synaptic pruning during CNS development describes the extensive synapse elimination that underscores activity-dependent refinement of neural circuits (Hua and Smith, 2004; Katz and Shatz, 1996; Lichtman, 1977; Low and Cheng, 2006; Sanes and Lichtman, 1999). The most extensively studied examples of activity-dependent synaptic pruning are development of the visual system and motor systems, which incorporates synapse disassembly in the cerebellum and at the neuromuscular junction (NMJ).

In the visual system, vast numbers of synapses are eliminated between thalamocortical axons and cortical layer IV neurons (Hubel et al., 1977), and in the cerebellum, inputs are lost between climbing fibers and Purkinje cells (Crepel et al., 1976; Lohof et al., 1996). Peripherally, populations of preganglionic synapses between partnering ganglion cells dismantle (Lichtman, 1977), and at the NMJ, selected motor axons disassociate from muscle fibers (Sanes and Lichtman, 1999). The systematic loss of synapses in all these cases is associated with the elaboration of synapses on the remaining axon(s), and in cases where axons completely disconnect from target cells, the target cells retain inputs from other neurons (Brown et al., 1976). Importantly, these studies demonstrate that synapse elimination is not a result of axon

degeneration or aberrant health of the target cell. Rather, selective synapse disassembly eliminates less active synapses and defines functional neural pathways.

- *The visual cortex*

In rodents, cats and primates, cortical neurons in layer IV of the visual cortex are tightly segregated into distinct columns, termed ocular dominance columns (ODCs), which are innervated by inputs from each eye to form eye-specific domains (Jaubert-Miazza et al., 2005; Shatz and Sretavan, 1986). ODCs are not present at birth; instead, inputs from both eyes are intermingled. During the first weeks of eye opening, synapses and axon arbors are systematically eliminated to achieve an accurate topographical map representing coordinates in the visual field that are characteristic of normal binocular adult connectivity patterns (Shatz and Sretavan, 1986). Loss of sensory input during early postnatal development, through occlusion of one eye, induces a loss of cortical response (Wiesel and Hubel, 1963); retinal afferents from the occluded eye rapidly disassemble and afferents from the open eye elaborate and colonize layer IV regions that would otherwise have been innervated by the occluded eye (Antonini and Stryker, 1993, 1996). Therefore, loss of activity induces synaptic disassembly. However, numerous anatomical and functional studies provide compelling evidence for activity-dependent synapse elimination in the developing visual system (Goda and Davis, 2003; Huberman, 2007).

Long-term *in-vivo* imaging studies of retinotopic map development in zebrafish and *Xenopus* larvae demonstrate disassembly of individual synapses selectively directs neurite branch dynamics *in-vivo* (Meyer and Smith, 2006; Niell et al., 2004; Ruthazer et al., 2003; Ruthazer et al., 2006). Synapse elimination precedes axon retraction, whereby axons regress to the nearest stable synapse (Meyer and Smith, 2006). NMDA-R antagonists selectively inhibit axon branch elimination along weakly innervated branches suggesting a mechanism of pruning less active axonal projections (Ruthazer et al., 2003). Ruthazer et al. show that in early retinotectal map development, correlated NMDA-R activity specifically regulates axon branch elimination rather than branch addition, and that these two opposing activities are concurrent. Together, these finding suggest incremental changes regulate and modify axonal arbor structure. These studies highlight the critical role of activity-dependent synapse elimination in establishing early CNS architecture.

In postnatal rodents, prior to eye opening, the retina exhibits robust and characteristic waves of spontaneous activity that drive action potentials through retinogeniculate cells into the thalamus and onto the primary visual cortex (Hanganu et al., 2006). Blockade of either early spontaneous activity prior to eye opening or deprivation of early visual experience once eye opening has occurred, reveal two distinct developmental phases in the formation and refinement of visual circuits (Hooks and Chen, 2006). First, spontaneous activity provides course eye specific territories within the thalamic lateral geniculate nucleus (LGN) by eliminating the majority of excessive afferents and strengthening the remaining synapses over the four-day period that spans eye opening (Hooks and Chen, 2006). Secondly, visual experience refines the circuits by

further synapse elimination and also by promoting maturation, maintenance and plasticity of remaining synapses (Hooks and Chen, 2006; Huberman, 2007).

Electrophysiological recordings during the three weeks that span eye opening in mouse, reveal that geniculate cells are initially innervated by at least 20 retinal inputs, by the end of the recording period only 1-3 imputs remain (Chen and Regehr, 2000). Blockade of retinal firing by intraocular injection of tetrodotoxin (TTX) during early development prevents the formation of ODCs due to defects in pruning and restriction of synaptic territories and terminal arbors, which instead remain extensively branched and overlapping (Antonini and Stryker, 1993; Hooks and Chen, 2006; Reiter et al., 1986; Stryker and Harris, 1986). Consistent with these data, NMDA-R antagonists disrupt experience-dependent synapse elimination in the visual cortex (Bear et al., 1990; Colonnese and Constantine-Paton, 2006; Kleinschmidt et al., 1987).

- *The neuromuscular junction*

At the rodent NMJ, acetylcholine receptors (AchRs) are initially dispersed in the postsynaptic muscle fiber. As incoming axon terminals approach the muscle fiber AchRs cluster to form a high-density plaque of receptors. Multiple terminal axons subsequently innervate receptors within individual plaques. Over the first few weeks of life, innervation becomes progressively restricted to a single axon in a protracted step-wise manner (Balice-Gordon et al., 1993; Colman et al., 1997). Initially, each of the multiple axons are anatomically indistinguishable and are capable of causing muscle contraction of equivalent strength, suggesting there is no initial bias or predetermination for a single axon to dominate at the onset of disassembly (Balice-Gordon et al., 1993; Brown et al., 1976; Colman et al., 1997). Intracellular recordings from newborn mouse muscle fibers suggest a cascade of pre- and postsynaptic changes mediate NMJ refinement; selected axon terminals start to release less neurotransmitter and the synapse become functionally weaker (Colman et al., 1997), before complete disassembly and axon withdrawal (Balice-Gordon et al., 1993). Importantly, the relative strength of remaining synapses subsequently increases, until finally, one axon terminal remains “victorious” (Balice-Gordon et al., 1993; Colman et al., 1997). Together, these data suggest competitive behavior between inputs at the NMJ.

At postnatal day 7 (P7) or P9 many rodent NMJs are in late phase disassembly and are innervated by only two axons (Kasthuri and Lichtman, 2003). The rate of synapse disassembly varies considerably between different NMJs, which was initially interpreted as disassembly being regulated by local mechanisms (Jansen and Fladby, 1990; Keller-Peck et al., 2001). However, tracing individual axon arbors from co-innervated NMJs at P7-9 reveals that individual neurons, or axon branches of a specific neuron determine synapse fate (Kasthuri and Lichtman, 2003). Neurons with smaller axon arborizations have a greater competitive advantage over neurons with larger more extensive axon arbors (Kasthuri and Lichtman, 2003). Furthermore, there is an inverse correlation between individual synapse size and competitive capacity; these findings suggest the role of neuron specific limiting factors in determining synapse fate (Kasthuri

and Lichtman, 2003). It is hypothesized that competitive advantage is determined by activity patterns of individual neurons and/or axon branches, and that observed asynchronous synapse disassembly along specific axon branches reflects protracted redistribution of synaptic components, which determines individual synapse efficacy (Kasthuri and Lichtman, 2003). Redistribution of synaptic components may eventually lead to entire axon braches having greater or lesser competitive advantages over co-innervated synaptic partners leading to the strengthening or weakening of axon branches. This hypothesis places neuron identity as the key factor in determining synapse fate.

Genetic manipulations that selectively inhibit neurotransmission demonstrate that more active synapses destabilize weaker opponents competing for the same target. Spatiotemporal depletion of choline acetyltransferase from just one or two axon terminals that are part of multiinnervated NMJs demonstrate inactive presynaptic terminals assemble synapses in the presence of active competitors, but fail to maintain them (Buffelli et al., 2003). Furthermore, relative synaptic efficacies induce changes in axon diameter, which may stabilize or destabilize entire axon branches. Strong inputs cause a loss of axon caliber in the weaker opponents with concomitant gains in their own caliber (Buffelli et al., 2003). Functional analyses of the NMJ reveal that localized AChR block within one section of the NMJ induce a gradual loss of AChRs, which is subsequently followed by presynaptic disassembly (Balice-Gordon and Lichtman, 1994). Importantly, when the complete NMJ is functionally blocked, there is no elimination; only when small regions are targeted is elimination observed, which suggests that activation of postsynaptic receptors synapses are required to mediate the removal of inactive synapses (Balice-Gordon and Lichtman, 1994). Studies of cultured embryonic *Xenopus* NMJs demonstrate that when a muscle cell is innervated by two motoneurons, asynchronous postsynaptic activation suppresses acetylcholine release from the later firing motoneuron further suggesting a postsynaptic mechanism for inhibiting presynaptic transmission (Dan and Poo, 1992; Lo and Poo, 1991). Two additional lines of evidence suggest that postsynaptic mechanisms may drive synaptic disassembly at the NMJ. Firstly, denervation, and therefore total loss of presynaptic activity, does not induce rapid postsynaptic disassembly (Rich and Lichtman, 1989), and secondly, the amplitude of postsynaptic potentials is reduced when opposed to relatively weaker axons indicating reduced AChR number or function (Colman et al., 1997).

Taken together, the evidence suggests that developmental NMJ disassembly is driven by competitive, postsynaptic mechanisms through activity-dependent signaling and availability of neuron specific limiting factors. However, live imaging of the mouse NMJ reveals that retraction of the presynaptic membrane can occur before evidence of postsynaptic disassembly (Walsh and Lichtman, 2003). Therefore, whilst postsynaptic activity may instigate synapse elimination through activity dependent processes, the cellular mechanisms that mediate synapse disassembly are not fully resolved, and whether synapse elimination is initiated pre- or postsynaptically remains unclear.

- *The cerebellum*

In the mature cerebellum, Purkinje cells (PCs) have a highly stereotyped pattern of innervation; they receive up to 200,000 physiologically weak inputs from parallel fibers, and in contrast just a single climbing fiber (CF), which is capable of activating voltage-gated calcium channels throughout the dendritic arbor (Hashimoto and Kano, 2005). During early postnatal cerebellar development, four or more CFs innervate each PC and supernumerary synapses are subsequently eliminated until mono-innervation is achieved (Crepel et al., 1982; Crepel et al., 1976; Lohof et al., 1996). In the mouse, each of the multiple CF inputs elicits equivalent but relatively weak EPSCs compared to the mature CF/PC synapse (Hashimoto and Kano, 2003). During the first 2-3 postnatal weeks, which is the period of CF synapse disassembly in mouse, the EPSC amplitude from evoked individual CFs become progressively weaker, whilst one or two synapses become relatively stronger (Hashimoto and Kano, 2005). Differential amplitudes have been detected at synapses that contain two inputs suggesting that a single CF is strengthened relative to the other(s), and predominates over its competitor(s) until the multiple, weaker inputs are eventually eliminated (Hashimoto and Kano, 2003; Mariani and Changeux, 1981). The progressive, step-wise disassembly and elimination of supernumerate synapses from multiinnervated synapses bears many similarities with the functional refinement and maturation on the NMJ described above.

Parallel fibers (PFs) derived from granule cells form synapses onto distal regions of PC dendrites, whereas climbing fibers (CFs) form synapses onto proximal PC dendrites. The well-characterized cerebellar mutant mice *reeler*, *weaver* and *staggerer* lack PF/PC synapses; importantly to this discussion, these mice also fail to eliminate supernumerate synapses between CFs and PCs (Eaton and Davis, 2003; Hashimoto and Kano, 2005). This phenotype is mimicked by ablation of granule cells by X-ray irradiation (Hashimoto and Kano, 2005), which suggests that granule cell inputs onto the distal dendrites of PFs participate in the elimination of CF/PC synapses in proximal dendrites. Furthermore, temporal ablation of granule cells reveals the absence of functional inputs from PFs only affects the latter stages of synapse elimination between CFs and PCs (Hashimoto and Kano, 2005); the early stages of synapse elimination up until P10 are unaffected indicating two distinct phases of synapse elimination, the later phase being activity dependent (Hashimoto and Kano, 2005).

Further support for activity-dependent synapse elimination in the cerebellum is demonstrated through studies of mGluR1 null mice (Hashimoto and Kano, 2005); mGluR1 localizes to PC spines opposed to PF and CF synaptic terminals (Lopez-Bendito et al., 2001). Importantly, PC specific expression of mGluR1 on the null background rescues impaired synapse elimination in a dose dependent manner (Ichise et al., 2000). Also, consistent with the evidence for distinct phases of synapse elimination, mGluR1 null mice exhibit normal regression of CF synapses until P10, whereupon the process is arrested (Hashimoto et al., 2001). Furthermore, downstream blockade of neural activity between mossy fibers and granule cells by chronic application of NMDA-R antagonists inhibits the late phase of synapse elimination between CFs

and PCs (Kakizawa et al., 2000). Mice that are deficient in GluRδ2, which is expressed selectively in PCs (Takayama et al., 1996), also fail to eliminate supernumerary CF/PC synapses (Kashiwabuchi et al., 1995). These mice form fewer parallel fibers synapses onto PCs, and in their absence CFs form ectopic synapses onto the distal dendrites (Hashimoto et al., 2001). CF/PC synapse elimination is also impaired in mutant mice deficient in protein kinase Cγ (PKCγ) (Kano et al., 1995). PKC is implicated in a number of neural functions including ion channel activity and synaptic transmission (Tanaka and Nishizuka, 1994). Together, these studies demonstrate distinct phases of synapse elimination, where the latter phase is regulated by activity-dependent mechanisms via mGluR1, GluRδ2 and PKC.

On the presynaptic side, mature CFs release glutamate from their terminal synapses through simultaneous fusion of multiple synaptic vesicles (Hashimoto and Kano, 2003; Wadiche and Jahr, 2001). Measurements of glutamate transients within the synaptic cleft reveal the probability of multivesicular release is significantly reduced at weaker CF synapses, compared to stronger synapses, and this reduction appears to be a determinant for synapse elimination (Hashimoto and Kano, 2003). Importantly, recordings of the mean quantal amplitude, which is a measure of postsynaptic receptor density, reveal the decay of presynaptic release occurs before changes in postsynaptic function (Hashimoto and Kano, 2003). CF/PC synapse elimination therefore involves pre- and postsynaptic mechanisms. A fine dissection of temporal and spatial synapse disassembly events has yet to be fully elucidated, and as such, it is not possible to conclude whether pre- or postsynaptic mechanisms drive CF/PC synapse elimination in the cerebellum.

Cellular and molecular mechanisms of synapse disassembly

Time-lapse imaging reveals that central excitatory synapses can disassemble within 90 minutes and dendritic spines are completely eliminated within a day (Grutzendler et al., 2002; Okabe et al., 2001a; Trachtenberg et al., 2002). In contrast, the half-life of many synaptic proteins far exceeds this time frame, for example AMPA-R and NR2 is 18-23 and 16 hours respectively (Huh and Wenthold, 1999). The relatively rapid loss of synapses therefore suggests that synapse disassembly is an active process, most likely to depend upon regulated mechanisms rather than a passive loss of synaptic proteins (Goda and Davis, 2003). Synapse elimination within the NMJ is considerably more protracted compared to central synapses, and studies reveal a time course of days for full disassembly (Goda and Davis, 2003; Walsh and Lichtman, 2003). Even so, synapses within the NMJ do not passively disassemble; rather specific mechanisms tightly regulate the elimination of supernumerate synapses, albeit over a longer period of time compared to synapses in the brain. As described above, overwhelming evidence supports a model for activity-dependent synapse disassembly. Here I will discuss the cellular and molecular mechanisms that regulate central and peripheral synapses disassembly.

- *Cellular mechanisms of synapse disassembly*

Contact separation between pre- and postsynaptic partners involve a loss of CAM interactions and the removal of molecules and mechanisms that otherwise confer structural stability. Cellular mechanisms that regulate synapse disassembly include inhibition of protein synthesis and activation of protein degradation, together with the removal of synaptic proteins such as receptors, CAMs and structural scaffold proteins and cytoplasmic proteins, and changes in actin dynamics. Importantly, the removal of such synaptic components occurs in a step-wise manner that is consistent with a model for organized synapse disassembly.

Presynaptic loss of synapsin and SV associated proteins is observed before loss of postsynaptic receptors during synapse disassembly at the *Drosophila* NMJ (Eaton et al., 2002). This study carefully details presynaptic disassembly and the authors report a stereotyped hierarchy of presynaptic dissolution that is initiated with dismantling of the presynaptic cytoskeleton, followed by the removal of SVs and the active zone and ultimately retraction of the presynaptic membrane (Eaton et al., 2002). These events are subsequently followed by disassembly of the postsynaptic apparatus (Eaton et al., 2002). Similarly in rat hippocampal cultures, rapid SV dispersal from presynaptic sites is a preliminary event associated with NMDA-R mediated synaptic depression and synapse disassembly (Hopf et al., 2002). These data support the hypothesis that presynaptic innervation provides instructive and maintenance signals to the postsynaptic compartment (Saitoe et al., 2001; Sanes and Lichtman, 2001).

During synapse disassembly at the vertebrate NMJ, structural changes within the pre- and postsynaptic apparatus appear concomitantly (Rich and Lichtman, 1989). However, functional studies reveal reduced amplitude of postsynaptic potentials prior to axon withdrawal (Colman et al., 1997). Postsynaptic loss of AchRs induces the oval plaque structure to change into a pretzel shaped structure (Balice-Gordon et al., 1993). Detailed immunohistochemical analyses from whole-mount muscle preparations following nerve-crush reveal the first postsynaptic molecules to disassemble are AchRs (Culican et al., 1998) and associated proteins such as Rapsyn and Utrophin, which stabilize and anchor AchRs to the plasma membrane and cytoskeleton (Culican et al., 1998). Subsequently, cytoskeletal proteins dismantle and are removed from the postsynaptic terminal at considerably slower rates compared to the removal of AchRs (Culican et al., 1998)). Lastly extracellular proteins associated with the basal lamina, such as NCAM are removed at even slower rates (Culican et al., 1998). The order of events reported reveal that on the postsynaptic side neurotransmitter receptors are first in line to destabilize, which suggests that loss of postsynaptic stimulation is an early event in postsynaptic synapse disassembly.

In central excitatory synapses, spine head volume is a strong indicator of postsynaptic function and ability to respond to presynaptic release; larger spines generally have more AMPA-Rs (Baude et al., 1995; Matsuzaki et al., 2004) and exhibit larger EPSPs (Matsuzaki et al., 2004). In acute hippocampal slices taken from P18 rat pups, rapid spine shrinkage is associated with earlier phases of LTD and occurs within 15 minutes of low frequency stimulation (Zhou et al.,

2004). Recordings taken over a 1-hour period following low frequency stimulation reveal progressive spine shrinkage that strongly correlates with reduced EPSCs and spine retraction (Zhou et al., 2004). Synapse loss can occur without spine shrinkage (Bastrikova et al., 2008; Becker et al., 2008) suggesting that shrinkage is not necessarily the trigger that drives synapse disassembly. Indeed, the two processes may be independent of each other (Bastrikova et al., 2008). Spine shrinkage and synapse separation may represent different mechanisms of modulating synaptic transmission.

Localized protein degradation of synaptic proteins via the ubiquitin proteasome system (UPS) is active at CNS and peripheral synapses and evidence supports a regulatory role for protein degradation during axon outgrowth, neurite pruning, synaptogenesis, synapse elimination and neurotransmission (Haas and Broadie, 2008). During *C.elegans* development, supernumerary synapses form between HSNL motoneurons and target muscle cells that subsequently undergo elimination via a protein complex containing E3 ubiquitin ligase, which is a core component of the UPS (Ding et al., 2007). The UPS is also active in destabilizing synapses during memory retrieval in the adult mouse brain (Lee et al., 2008a). In a fear-conditioned context, memory retrieval involves reorganizing synapses to incorporate new experiences; this process involves destabilizing activated synapses through polyubiquitination (Lee et al., 2008a). Furthermore, in cultured neurons NMDA-R activation regulates proteasome mobilization between the dendritic shaft and spines to locally regulate postsynaptic protein composition (Bingol and Schuman, 2006; Guo and Wang, 2007) and thus provides a possible mechanism for activity-dependent protein degradation and synapse stability.

Postsynaptic blockade of protein synthesis by intracellular injection of ribosomal inactivating proteins at the NMJ mimics the stereotyped pattern of progressive synapse elimination that occurs during mammalian postnatal development (McCann et al., 2007). In this study, step-wise axon retraction occurred within 12 hours of muscle injection whilst the muscle itself remained functionally healthy; suggesting that synthesis of a maintenance signal was inhibited. Conclusive identification of molecules has yet to be obtained, but candidates include trophic factors such as BDNF, protease inhibitors and/or adhesion molecules (McCann et al., 2007). Whilst it is not clear whether protein synthesis is actively blocked during synapse elimination under physiological conditions, these data are consistent with the hypothesis for retrograde signaling at synapses (Tao and Poo, 2001) and that loss of trophic signaling across the synapse induces synapse elimination (Sanes and Lichtman, 1999).

- *Molecular mechanisms of synapse disassembly*

In principle, synapse disassembly can be stimulated by loss of a maintenance or trophic factor, or activation of a mechanism that drives disassembly (Eaton and Davis, 2003). Neurotrophins are widely reported to regulate synaptic maintenance at both peripheral and central synapses (McAllister et al., 1999; Sanes and Lichtman, 2001). At the NMJ, a model has been proposed whereby postsynaptic AchR activation stimulates the release of both neurotrophic and

neurotoxic factors that stabilize and protect the presynaptic counterpart, and destabilizes adjacent or nearby inactive synapses (Eaton and Davis, 2003; Jennings, 1994; Lichtman and Colman, 2000; Sanes and Lichtman, 1999). BDNF was recently identified as a candidate molecule that could satisfy this model as it acts as both a positive and negative retrograde signal (Yang et al., 2009a; Yang et al., 2009b).

BDNF is synthesized in a precursor form (pro-BDNF) that is subsequently cleaved by matalloproteases into mature BDNF (mBDNF) (Chao and Bothwell, 2002; Yang et al., 2009a). These two isoforms bind to different receptors; pro-BDNF preferentially interacts with the pan-neurotrophic receptor p75 ($p75^{NTR}$), whereas mBDNF binds with TrkB receptors (Chao and Bothwell, 2002). Critically, these two receptors signal to affect opposing outcomes, TrKB is critical to LTP (Lu et al., 2005; Poo, 2001; Rosch et al., 2005; Woo et al., 2005), whereas $p75^{NTR}$ activation regulates LTD (Yang et al., 2009a). Acute application of mBDNF activates presynaptic TrkB receptors at the NMJ inducing a rapid increase of neurotransmitter release (Lohof et al., 1993), and chronic application promotes NMJ maturation (Wang et al., 1995). In contrast, exogenous cleavage-resistant proBDNF applied to the NMJ suppresses both the frequency and amplitude of spontaneous and evoked synaptic currents and induces axon retraction and synapse disassembly through activation of $p75^{NTR}$ (Yang et al., 2009a).

proBDNF has been detected in the mouse hippocampus with strong expression during postnatal week two and ongoing low-level expression continuing into adulthood, which coincides with the main period of postnatal synaptic pruning and is consistent with adult synapse disassembly and LTD (Yang et al., 2009b). proBDNF temporal and spatial expression patterns coincide with p75 receptor expression suggesting a role for BDNF/p75 signaling in synapse disassembly (Yang et al., 2009b). This study also demonstrates that activity induces the release of proBDNF from cultured hippocampal neurons. Whilst the authors did not report on BDNF/p75 signaling as a regulator of central synapse disassembly, three months after the publication of this manuscript, the same group published data for proBDNF mediated synapse disassembly and LTD at the NMJ (as described in the preceding paragraph) (Yang et al., 2009a).

Other neurotrophins such as NT4 also interact with TrkB receptors to modulate synaptic maintenance in the CNS and PNS and their withdrawal or blockade induces synapse disassembly (Cohen-Cory, 2002; Huang and Reichardt, 2001; McAllister et al., 1999). Loss of either NT4 or TrkB at the NMJ induces synapse elimination (Belluardo et al., 2001; Gonzalez et al., 1999) and inhibition of TrkB signaling inhibits OCD formation during the critical period (Cabelli et al., 1995). However, whilst NT4 null mice display NMJ defects, *in-vivo* TrkB ablation does not affect synapse number after synapse formation (Luikart et al., 2005) suggesting possible redundancy between postsynaptic receptors.

The critical period of synapse pruning in the visual system coincides with the appearance of astrocytes (Christopherson et al., 2005; Ullian et al., 2001). Evidence reveals that purified murine retinogeniculate cells (RGCs) exposed to astrocytes upregulate the protein C1q

(Stevens et al., 2007). C1q is a subcomponent of the C1 complex within the classical pathway of the complement system, which is part of the innate immune system (Kishore and Reid, 2000). C1q binds with immunoglobulin G or immunoglobulin M and plays a key role in recognizing immune complexes and triggers a cascade of immunity, which in the CNS leads to the tagging of synapses and their elimination (Kishore and Reid, 2000; Stevens et al., 2007). Importantly, C1q is required during normal development of the visual system (Stevens et al., 2007). Punctate C1q commonly localizes with small SV2 or PSD-95 puncta that lack an opposing partner, and less frequently with larger puncta that are positive for both SV2 and PSD-95 (Stevens et al., 2007). This finding suggests C1q is present at synapses that are either immature or are in the progress of disassembly. C1q expression is developmentally upregulated between P4 and P10, and is then significantly downregulated at P15, and by P30 only very low expression levels are detected by immunohistochemistry (Stevens et al., 2007). The findings from this study suggest that immature astrocytes, which exist in the CNS until about P14, release a factor that stimulates C1q expression in RGCs. It is posited that downstream mechanisms of C1q mediated synapse elimination involve membrane phagocytosis by microglia (Stevens et al., 2007). This study reports a novel role for glia cells in mediating synapse disassembly. Whilst this study focused on the developing visual system, there is evidence that the complement cascade is also active in cortical regions and the hippocampus during the peak of synaptogenesis and synaptic pruning (Dalmau et al., 1998; Fiske and Brunjes, 2000; Maslinska et al., 1998; Stevens et al., 2007). C1q is also significantly upregulated in human Alzheimer's disease, amyotrophic lateral sclerosis and mouse models of glaucoma (Dangond et al., 2004; Fonseca et al., 2004; Stasi et al., 2006; Stevens et al., 2007). This finding is especially interesting as these diseases, and many other neurodegenerative disorders (as described below), are hallmarked by widespread synapse disassembly (Selkoe, 2002).

The disruption of binding between synaptic CAMs and dismantling specific scaffold proteins such as PSD-95 and/or the cytoskeleton would provide effective mechanisms for rapid synapse disassembly in mature neurons, and indeed do occur during the disassembly process characteristic during refinement. CAMs, scaffolding proteins and the cytoskeleton organize and maintain synapse structure yet remain remarkably dynamic in order to support and facilitate function (as described in Chapter 1.2). Electronmicrographs of the *Drosophila* NMJ reveal a clearing of electron dense material from the synaptic cleft and an increase in the size of the cleft during synapse disassembly suggesting a loss of transynaptic complexes and adhesion proteins (Eaton and Davis, 2003). To date it has not been elucidated whether specific molecules are activated to directly attack the integrity of these structural complexes during disassembly, or alternatively whether limited supply of a maintenance factor released in an activity dependent manner could destabilize the synapse sufficiently to induce disassembly. It is clearly evident from the above discussion that the nature of the molecular mechanisms underlying synapse disassembly is poorly understood, and the relationship between delivery of synaptoxins and withdrawal and blockade of trophic/maintenance factors have yet to be elucidated.

Synapse disassembly in the young and adult brain

It is generally accepted that experience and learning modulate neural connectivity in the brain (Alvarez and Sabatini, 2007; Holtmaat and Svoboda, 2009; Katz and Shatz, 1996). Principally, there are two models that may explain how sensory experience is encoded in the brain (Alvarez and Sabatini, 2007); firstly, new synapses assemble temporally and spatially in direct response to stimulus, which generates neural connections that reflect meaningful and relevant information (Alvarez and Sabatini, 2007). Secondly, synapse assembly maybe constitutive, and activity-dependent synapse disassembly eliminates redundant synapses, thus preserving only meaningful connections. Alternatively, experience-led circuit modifications may be a combination of these two models, together with subtle modulations in synaptic structure such as synapse strengthening and weakening (Holtmaat and Svoboda, 2009).

Long-term *in-vivo* imaging of spines in the mouse somatosensory cortex reveals that spines appear and disappear at a rapid rate during the first two weeks of life (Holtmaat et al., 2005; Lendvai et al., 2000). The rate of synapse turnover subsequently reduces with age, presumably as synapses mature and circuits stabilize (Holtmaat et al., 2005; Nagerl et al., 2004; Trachtenberg et al., 2002). In the adult mouse brain under baseline conditions, approximately 70-90% of the synapse population is stable (De Paola et al., 2006; Grutzendler et al., 2002; Holtmaat et al., 2005; Majewska et al., 2006; Trachtenberg et al., 2002; Zuo et al., 2005a). However, studies have shown that *de novo* synapses form in response to novel sensory experiences yet the net number of synapses remains relatively constant, which implies a concomitant and coordinated process of synapse disassembly (Holtmaat et al., 2008; Holtmaat et al., 2005; Trachtenberg et al., 2002). Approximately 5% of the newly formed synapses are integrated into the stable population (Holtmaat et al., 2008). Research has yet to elucidate where synapses are lost to accommodate for this increase and how activity dependent changes in synapse turnover impact on structural plasticity within the adult brain. Different cell-types display significantly different capacities for assembling and disassembling synapses (Holtmaat and Svoboda, 2009), and this presents an enormous challenge to understanding how activity dependent structural modifications occur across established neural networks.

In the mouse somatosensory cortex, long-term *in-vivo* imaging studies of spines demonstrate that sensory deprivation, through whisker trimming, significantly reduces the rate of synapse elimination in adolescent mice, and continues to do so into adulthood albeit to a lesser extent (Zuo et al., 2005b). Importantly, the rate of synapse loss is rescued when the whiskers are allowed to regrow and sensory experience is restored (Zuo et al., 2005b). Furthermore, the loss and rescue effects on the rate of spine elimination are mimicked by delivery and withdrawal of NMDA antagonists (Zuo et al., 2005b). High-frequency stimulation enhances the rate of synapse turnover in mature hippocampal slice cultures through activation of AMPA-R, PKA and protein synthesis (De Paola et al., 2003). Here, the authors suggest the highly dynamic behavior of presynaptic terminals within the mature hippocampus represents competition for

innervation of postsynaptic spines reminiscent of synapse elimination during developmental synaptic pruning.

Activity-dependent mechanisms induce synapse shrinkage and disassembly in mature neurons through LTD (Becker et al., 2008; Nagerl et al., 2004; Zhou et al., 2004). LTD is electrophsiologically detected as a decline in synaptic transmission and requires activation of NMDA-Rs (Dudek and Bear, 1992; Mulkey and Malenka, 1992). LTD is induced by low frequency synaptic stimulation and also follows LTP induction (Artola et al., 1990; Bramham and Srebro, 1987). LTP and LTD are understood to be partnering mechanisms in synaptic plasticity that regulate responsiveness of the pre- and postsynaptic cell following excitation, by modifying presynaptic release and the threshold for postsynaptic firing (Dudek and Bear, 1992; Ito, 1989; Malenka and Bear, 2004). LTD induction in the developed hippocampus (17 DIV organotypic slices prepared from P5-7 mice pups) significantly increases the turnover of presynaptic boutons, resulting in a net loss of synaptic contacts, during a 4-hour recoding period after LTD induction, as detected by time-lapse imaging of Synaptophysin-GFP, calcium imaging and retrospective electronmicroscopy (Becker et al., 2008). A significant proportion (33%) of lost synaptic contacts were due to a retraction of presynaptic boutons, where the associated spine persisted without evidence of any gross morphological changes. In cases where spine retraction preceded bouton changes and loss of contact, the counterpart boutons became rapidly smaller in volume following spine shrinkage (Becker et al., 2008). This study reveals a potential presynaptic mechanism for modulating synapse disassembly that could initiate elimination, and reveals a rapid presynaptic response to postsynaptic changes. Importantly, the structural changes observed in this study were observed after 2-hours after LTD expression, which suggests that other mechanisms mediate early phases of LTD. The possibility of two distinct phases regulating synapse disassembly in the developed hippocampus is in parallel with the model discussed for synapse elimination during cerebellar development. The experiments described within this section compellingly demonstrate that activity-dependent mechanisms induce synapse shrinkage and disassembly. These synaptic modifications alter functional efficacy of synaptic transmission and hold the potential to affect structural changes within neural circuits.

Synapse disassembly as a model for neurodegenerative disease

Reduced synapse number correlates with mental retardation, schizophrenia, and other developmental disorders (Glantz and Lewis, 2000; Kaufmann and Moser, 2000; Selkoe, 2002). However, it is unclear in such developmental disorders whether the reduced number of synapses is due to a failure of synapse maturation or an increase in synapse disassembly. Evidence demonstrates that smaller immature spines are more prone to losing presynaptic contact compared to larger more mature spines (Bastrikova et al., 2008). Therefore, deficits in spine maturation could impact significantly on synapse number and subsequent adult neural circuitry.

Compelling evidence suggests that synapse disassembly, as a result of synaptic dysfunction, may be the stimulus for widespread neuronal degeneration that hallmarks neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's, Huntington's, amyotrophic lateral sclerosis and glaucoma (Arendt, 2009; Day et al., 2006; Stevens et al., 2007; Yoshiyama et al., 2007). One of the earliest symptoms of AD is the inability to encode new memories, and as the disorder advances declarative and nondeclarative memory becomes progressively impaired, as does the capacity for reasoning, abstraction and language (Selkoe, 2002). The protracted loss of selective neural function in the AD brain may reflect progressive synapse dysfunction within cholinergic and glutamatergic circuits (Selkoe, 2002; Small et al., 2001). Biopsied human cortices, within 2-4 years of clinical AD onset, reveal a ~15-35% loss of synapses per neuron (Terry et al., 1991) and the degree of cognitive decline correlates with loss of synaptophysin immunoreactivity (Masliah et al., 2001). Amyloid-β (Aβ) plaques are defining brain lesions of the AD brain, and evidence supports a causal role for Aβ plaques in AD progression (Selkoe, 2002; Wang et al., 1999). However, bath application of physiological concentrations of soluble Aβ oligomers onto organotypic hippocampal cultures reveals significant loss of synapses in the absence of plaque formation (Shankar et al., 2007). Furthermore, mutant mouse models of AD reveal the number of synaptophysin puncta is significantly reduced (~30%) (Hsia et al., 1999), and synaptic transmission and/or LTP is decreased in the absence of Aβ plaque formation (Selkoe, 2002). In these mutant mice models, the synaptic deficits recorded correlate with elevated levels of diffusible Amyloid β protein, which may operate as a synaptotoxic factor, before aggregating into Aβ plaques. Together, these studies strongly suggest that synapse dysfunction and elimination, through exposure to Aβ are early mechanisms underlying the insidious loss of neurons in the AD brain. Further research will reveal if other, disease specific synaptotoxins, deleteriously affect synapse function and induce synapse disassembly.

Summary of synapse disassembly

Synapse disassembly is tightly regulated both temporally and spatially and is mediated by local changes at the level of individual synapses. Together with synapse assembly, synapse elimination modulates structural plasticity within neural circuits during development and throughout adult life. There are three main contexts in which synapses disassemble; firstly as part of the postnatal program of circuit refinement; secondly in the mature brain as a modulator of synapse and/or circuit remodeling; and thirdly in the diseased brain as a putative trigger for neurodegeneration.

Different time courses of synapse disassembly have been described and it remains unclear whether this reflects different stages of neuronal development. For example, following induction of LTD in mature synapses there is a 2-hour lag before indications of presynaptic disassembly (Becker et al., 2008), whereas younger cultures show spine retraction after just 15-30 minutes (Bastrikova et al., 2008). Do these different timeframes reflect differences in pre- and postsynaptic mechanisms? As described, it is clear that pre- and post synaptic mechanisms are deployed during synapse disassembly, however different research groups have described

different orders of pre- and post disassembly, and it is not clear whether there is a strict stereotyped hierarchy of synapse disassembly in terms of which is the first to disassemble, or indeed which compartment actually precipitates disassembly.

Importantly, synapse disassembly is not a passive process. Rather, pre and postsynaptic mechanisms collaborate in the loss of synaptic junctions in an activity dependent manner. Studies of cerebellar synapse elimination suggest early and late phases of disassembly, which probably represent deployment of different mechanisms, with the later phase being activity-dependent. This raises the question of whether the early phase could be regulated by availability and competition for trophic factors. As described, the withdrawal or blockade of trophic factors alone may not be sufficient to induce rapid synapse loss, and it is probable that a process of destabilization and protein degradation are also required. Given that synapses are intrinsically dynamic structures, the blockade of stabilization or maintenance mechanisms together with withdrawal of trophic factors or exposure to a synaptotoxin may cooperate in the dissolution of synaptic structures.

Detailed analysis of synapse disassembly at the molecular level has yet to be fully elucidated. The different timeframes of synapse disassembly may reflect developmental stages of the synapse indicating that younger synapses are more susceptible to disassembly, possibly due to reduced levels of CAMs and/or other related molecules that maintain the structural integrity. On the other hand, the timeframe variance may indicate different mechanisms deployed at different stages or different paradigms. Whilst a number of mechanisms and candidate molecules have been proposed, a detailed account of synapse disassembly at different developmental stages has yet to be completed and until then many issues remain poorly understood. It is somewhat surprising that the study of mechanisms underlying synapse disassembly in the brain is so far behind research into synapse assembly, especially given its great importance in the development of functional neuronal circuits and circuit plasticity, in addition to links to neurodegenerative disorders and the potential for translational research.

1.3.3.Synapse maintenance

Once synapses are assembled and physiologically functional they are either maintained or disassembled. Long-term in-vivo imaging has demonstrated that 70-90% of synapses in the mature brain are stable and persist for extended periods of time, perhaps for the lifespan of the animal (Alvarez and Sabatini, 2007; Bhatt et al., 2009; Holtmaat and Svoboda, 2009; Meyer et al., 2003; Trachtenberg et al., 2002). Synaptic function and maintenance in the CNS not only determines the passage of information through the brain, but also establishes and maintains axonal and dendritic arbors, which in turn determines the structural architecture of the brain (Chen et al., 2010; Hu et al., 2005; Meyer and Smith, 2006; Niell et al., 2004; Rajan et al., 1999; Ruthazer et al., 2006; Wu and Cline, 1998). *In-vivo* analyses reveal motile “exploratory” axon branches form preferentially at nascent presynaptic sites; in contrast stable presynaptic sites inhibit dynamic axon behaviors and growth and are associated with stable axon branches

(Meyer and Smith, 2006). These studies are supported by the finding of a direct correlation between synapse maturation and local axon stability, which is enhanced by neuronal activity (Ruthazer et al., 2006). The relationship between synaptic and axonal maturation and stability is equally reflected in dendritic arbor structure, where loss of synapses is associated with dendritic retraction (Lin and Koleske, 2010; Sfakianos et al., 2007). Together, the evidence suggests that synaptic maturation and maintenance is critical for neurite stability and preservation of neuronal networks.

Activity dependent mechanisms stimulate both synapse assembly and disassembly (as described in the preceding chapters 1.3.1 and 1.3.2). Consistently, abundant evidence suggests that neuronal activity regulates synaptic maintenance. The Canadian psychologist Donald Hebb first postulated a role for activity driven mechanisms in determining and maintaining functional neural circuits in his pioneering book “The organization of behavior; a neuropsychological theory” in 1949 (Hebb, 1949). His theory is often summarized by the well-known phrase “Cells that fire together, wire together”; for a Perspective on Hebb’s legacy see (Brown and Milner, 2003). As will become evident from the discussion below, Hebb’s postulate has stood the test of time.

In contrast to the longevity of synapses in the mature brain, studies indicate that synaptic proteins are highly labile with half-lives of just days or less (Ehlers, 2003; Huh and Wenthold, 1999). Furthermore, synaptic proteins such as Ras, Shank, PSD95 Bassoon and Synaptophysin dynamically redistribute between adjacent synapses over a time scale of minutes and hours (Gray et al., 2006; Kim and Sheng, 2004; Tsuriel et al., 2009; Tsuriel et al., 2006). These studies raise a fundamental question; if synaptic components are only transiently located to synaptic sites, how is long term synaptic integrity maintained?

Presynaptic activity and synapse stability

Action potentials stimulate neurotransmitter release from the presynaptic terminal in a calcium-dependent manner (as previously described). Munc18 (mouse uncoordinated-18 protein) is an essential component of the synaptic vesicle fusion machinery that regulates SV exocytosis through its interactions with SNARE complexes and is required for regulating calcium-dependent neurotransmitter release (Bouwman et al., 2004; Garcia et al., 1994; Hata et al., 1993b). *In-vivo* studies of mutant mice deficient in Munc18 demonstrate a critical role for presynaptic activity in stabilizing and maintaining newly formed synapses. These mice display deficits in evoked and spontaneous neurotransmitter release (Bouwman et al., 2004). Whilst the mutant mice die shortly after birth, embryonic analyses by electron microscopy at E16 reveal that both mutants and wild-type littermates form immature synapses with equivalent morphologies suggesting the initial process of synapse assembly is not adversely affected (Bouwman et al., 2004; Verhage et al., 2000). Whilst *Munc18* mutant mice present fewer synapses at E16, the deficit in synapse number becomes progressively more significant over the following two days and by E18 mutant synapses are relatively sparse (Bouwman et al.,

2004). The significant decrease in synapse number in *Munc18* mutant mice suggest that existing synapses are being lost (Bouwman et al., 2004). Furthermore *Munc18* mutant synapses contain more pleimorphic vesicles and multivesicular structures within the pre- and postsynaptic compartments, which may form from unstable synaptic components and is indicative of synapse disassembly (Bouwman et al., 2004). These findings suggest that presynaptic release of neurotransmitter and/or a neuromodulatory factor is required for maintaining synapses in the developing brain (Bouwman et al., 2004).

Time-lapse imaging of Synaptophysin-GFP reveals that postsynaptic surface expression of the AMPA-R sub-unit GluR2 stabilizes presynaptic inputs (Ripley et al., 2010). In these experiments, the researchers differentiated between AMPA-R activation and AMPA-R availability by using pharmacological antagonists or manipulating receptor expression respectively. They found that loss of AMPA-R expression from the postsynaptic membrane destabilizes presynaptic Synaptophysin-GFP clusters but functional blockade of AMPA-Rs did not. Importantly, they establish the N-terminal domain of GluR2 (GluR2 NTD), which extends into the synaptic cleft, is sufficient for presynaptic stabilization. Furthermore, overexpression of GluR2 NTD and Neurologin1 in Human Embryonic Kidney 293T cells significantly enhances the stability of neuronal Synaptophysin-GFP clusters formed between neurons and cell lines (Ripley et al., 2010). These results suggest an instructive role for AMPA-Rs in maintaining synapse structure, whereby the extracellular domain of GluR2 provides structural interactions, possibly via the cell-adhesion molecule Neuroligin1, to regulate presynaptic stability and maintenance. Although interactions and identification of binding domains between GluR2 NTD and Neuroligin1, or the presynaptic mechanism have yet to be elucidated, these data provide a framework for a transynaptic mechanism that regulates the stability between postsynaptic receptors and cell adhesion molecules (Ripley et al., 2010).

Activity-dependent trafficking of postsynaptic molecules

PSD95 is the core postsynaptic scaffold protein that clusters and organizes receptors and associated signaling molecules at the PSD, and as such is an important regulator of maintaining synapse protein composition and stability (Ehrlich et al., 2007; El-Husseini et al., 2000; Elias and Nicoll, 2007; Kim and Sheng, 2004). Acute knockdown of PSD-95 by RNAi reduces the number of stable spines, and concomitantly increases the number of transient spines and the rate of spine turnover (Ehrlich et al., 2007). *In-vivo* analyses demonstrate that PSD95 is highly dynamic under basal conditions despite maintaining a persistent pool (Gray et al., 2006). Tracking of PSD95 in the barrel cortex of mice (postnatal day 10-21) by two-photon activation of photo-activatable PSD95-GFP reveals that synaptic PSD95 exchanges with PSD95 from nearby synapses by a process of lateral diffusion (Gray et al., 2006). This study reveals that synaptic PSD95 has an average (median) half-life of approximately just 60 minutes; PSD95 retention time at the synapse increases as the animal matures but rarely exceeds 200 minutes.

The retention time of photoactivatable PSD95-GFP at individual synapses is primarily determined by interactions with binding partners within the PSD (Gray et al., 2006) such as GKAP, Shank, PSD-Zip45 and CaMKII (Kuriu et al., 2006). Once PSD95 is released from the PSD it rapidly diffuses through the dendrite and becomes incorporated into neighboring PSDs, suggesting a process of recycling until it is finally degraded. PSD95 has a half-life of ~36 hours (Ehlers, 2003; El-Husseini Ael et al., 2002). Gray et al. report a correlation that larger spines are more efficient at capturing and retaining diffusible PSD95 compared to smaller PSDs. This linear relationship between spine size and PSD95 capture and retention suggests the presence of a mechanism for maintaining synapse size (Gray et al., 2006). These data underscore the remarkably dynamic state of synapses and reveals a process of rapid protein trafficking in and out of the PSD to maintain synaptic stability and size. Importantly, sensory deprivation of adult mice (60 days old) by whisker trimming dramatically reduces the retention of PSD95 at synaptic sites, which strongly indicates that sensory activity is required to maintain a persistent postsynaptic scaffold structure (Gray et al., 2006).

A number of *in-vivo* and *in-vitro* studies have demonstrated postsynaptic receptor trafficking in and out of the PSD by activity driven mechanisms (Ehlers, 2000; Ehlers et al., 2007; Kielland et al., 2009; O'Brien et al., 1998; Rao and Craig, 1997). Together, the evidence suggests that receptor recycling through endo- and exocytosis, and lateral diffusion all contribute to receptor trafficking at the synapse, which dynamically maintains a pool of receptors at the PSD (Cognet et al., 2006; Collingridge et al., 2004; Malinow and Malenka, 2002; Wenthold et al., 2003). In the visual system, three distinct pools of AMPA-Rs have been identified at retinogeniculate synapses, the residual pool (>100 nm from the postsynaptic membrane), the deliverable pool (~30-100 nm from the postsynaptic membrane) and the synaptic pool (within the PSD) (Kielland et al., 2009). By combining electrophysiological approaches with immuno-electronmicroscopy, the authors demonstrate that activity-dependent stimulation maintains a population of available AMPA-Rs by driving the vertical movement of AMPA-Rs from the deliverable pool to the synaptic pool by signaling through the small GTPase Ras and CaMKII (Kielland et al., 2009).

Lateral diffusion of the GluR1 AMPA-R subunit has been demonstrated using quantum dots conjugated with GluR1 antibody (GluR1-QDs), which combined with rapid time-lapse imaging enables single molecule tracking (Ehlers et al., 2007). Here, the researchers selectively inactivated presynaptic release by coexpression of Synaptophysin-GFP and tetanus toxin light chain. The data revealed GluR1 containing receptors diffuse through synapses that are presynaptically inactive, but are selectively captured and stabilized at neighboring active synapses (Ehlers et al., 2007). Accordingly, individually silenced synapses contain less GluR1 compared to neighboring active synapses (Ehlers et al., 2007; Harms and Craig, 2005). By plotting the fine movement of GluR1-QDs at high resolution, the analyses reveal that at inactive synapses GluR1 appears to freely and randomly explore the postsynaptic membrane. At active synapses, GluR1 diffusion is tightly restricted to ~20% of the synaptic membrane with further stabilization domains of ~50 nm (Ehlers et al., 2007). This suggests that presynaptic activity reorganizes the postsynaptic membrane into sub-domains, which present restraints against

diffusion out of the PSD (Ehlers et al., 2007). The authors discuss that physical restraints against diffusion could be provided by cytoskeletal rearrangements, the presence of large protein complexes and/or changes in protein-protein interactions. Further research is required to determine how presynaptic activity instructs changes within the PSD architecture or influences biochemical properties. This hypothesis of diffusional trapping differs from current models of active receptor recruitment to the PSD in response to stimulation (Kopec et al., 2006; Park et al., 2004; Passafaro et al., 2001; Shi et al., 2001). However, the Ehlers *et al.* (2007) study may provide evidence for a two-step process of AMPA-R delivery to synaptic sites, whereby local synaptic activity couples AMPA-R exocytosis to perisynaptic zones with lateral diffusion into the PSD. Importantly, this study elegantly demonstrates a role for presynaptic activity in locally regulating the mobilization and maintenance of a pool of GluR1 at postsynaptic sites possibly by structurally reorganizing the PSD.

Cell-adhesion molecules

It is reasonable to speculate that CAMs are important regulators of synaptic maintenance as they provide transynaptic adhesion and stability, and a platform for bidirectional synaptic signaling. However, understanding the role and function of CAMs (see chapter 1.2.1.) does not automatically implicate their role as modulators of long-term synaptic maintenance. The presence of multiple CAMs at individual synapses (Figure 1.2) has made it difficult to determine the role of individual CAMs. However, recent studies on Neuroligin/Neurexin (NRX/NLG) and Cadherin complexes shed new light on how these complexes participate to confer activity-dependant synaptic maintenance and the development and stabilization of neural circuits.

N-cadherin is present at mature synapses and in accordance with other CAMs, signals bidirectionally to regulate and coordinate pre- and postsynaptic function (Bruses, 2006; Kwiatkowski et al., 2007). Presynaptically, the cytoplasmic tail of N-cadherin binds to β-catenin, which is involved in recruiting SVs through PDZ domain interactions (Bamji et al., 2003; Perego et al., 2000). These interactions are required to maintain a functional pool of presynaptic SVs and thus contribute to efficient neurotransmitter release (Bamji et al., 2003). β-catenin also regulates actin polymerization by signaling through small GTPases and multiple actin-binding proteins to regulate stability (Bamji, 2005). Postsynaptically, N-Cadherin binds with α-Catenin to regulate spine stability (Abe et al., 2004; Okamura et al., 2004; Togashi et al., 2002). α-Catenin binds to and bundles F-actin and provides a critical link between the plasma membrane and cytoskeleton (Rimm et al., 1995). Overexpression of α-Catenin reduces spine motility and increases the number of stable contacts with axons *in-vitro* (Abe et al., 2004). Consistently, blockade of α-Catenin function by expression of a dominant-negative or targeted mutation of α-Catenin reduces the number of stable spines and increases the number motile spines that lack presynaptic contact (Abe et al., 2004; Togashi et al., 2002). Furthermore, neuronal activity induces *cis*-dimerization of N-caderins in the plane of the synaptic membrane, which may provide further mechanisms for stabilizing synaptic contacts (Bruses, 2006) and enhancing synaptic transmission (Tanaka et al., 2000).

Two recent studies have identified a tripartite complex of presynaptic Neurexin, postsynaptic GluRδ2 and the secreted protein Cerebellin1 (Cbln1) (Matsuda et al., 2010; Uemura et al., 2010) that mediates bidirectional synaptic stability and maintenance (Yuzaki, 2011). Cbln1 is a member of the C1q family, which was previously described as a regulator of synapse elimination (Stevens et al., 2007) (see chapter 1.3.2 Synapse disassembly). In contrast, this family member is characterized as a synaptogenic factor and synaptic "organizer" in the cerebellum (Yuzaki, 2011). Cbln1 is secreted by granule cells and enriched within the synaptic cleft at parallel fiber-Purkinje cell synapses; Cbln1 mRNA is also localized to specific regions of the brain including the olfactory bulb, entorhinal cortex and thalamic nuclei (Yuzaki, 2011). Microinjection of recombinant Cbln1 into the cerebellum rescues the ataxia phenotype presented by Cbln1 null mice and maintains a normal phenotype until the injected Cbln1 is degraded (Ito-Ishida et al., 2008). Furthermore, ectopic Cbln1 on a null background reversibly rescues synapse number and function *in-vitro*. Together, these studies suggest that Cbln1 not only stimulates the formation of synapses but it's continued presence is required to functionally maintain them (Ito-Ishida et al., 2008). Cbln1 binds with the extracellular domains of GluRδ2 and Neurexin1β to form a transynaptic complex that is insensitive to extracellular Ca^{++} concentration, which consistent with a role for robust cell adhesion (Yuzaki, 2011). Furthermore, Neurexin and GluRδ2 bind with the pre- and postsynaptic scaffolding proteins Cask and Shank2 respectively (Yuzaki, 2011), which further facilitates the role of this complex in maintaining synaptic structure.

The stabilization of developing dendritic arbors occurs concomitantly with synapse assembly and stability (Cline and Haas, 2008; McAllister, 2007; Wong and Ghosh, 2002) and requires the formation of transynaptic complexes of Neurexin1β and Neuroligin1 (NX1β and NL1 respectively) (Chen et al., 2010). By using rapid two-photon time-lapse imaging together with 3D quantification of dendritic growth behaviors, Chen *et al.* identify populations of dendritic filopodia as nascent dendritic branches (as opposed to precursor spine structures). Importantly, the authors show that local infusion of a dominant-negative Neurexin1β construct (dn-NX) ablates synaptic NX1β/NLG1 interactions and significantly reduces the number of functional synapses and the number of existing stable dendritic filopodia *in-vivo* (Chen et al., 2010). Furthermore, ablation of NX1β/NLG1 interactions, either by dn-NX or introduction of NL-1 morpholino reduces the formation of new stable dendritic filopodia, which in turn impedes the development of complex dendritic arbors (Chen et al., 2010). The findings indicate that cell adhesion through NX1β/NLG1 interactions stabilize nascent synaptic sites *in-vivo*, which when coupled with NMDA-R activation stabilizes the cytoskeleton to prevent synapse loss and filopodia retraction (Chen et al., 2010). This study highlights the importance of synapse stability for dendritic branch stability and the subsequent development and elaboration of neural circuits. Whilst it is evident that certain CAMs support synaptic maintenance, considerable research is still required to understand how CAMs functionally cooperate in the mature brain to regulate synapse stability, and how the balance of structural stability and flexibility is achieved to enable synaptic plasticity.

Secreted factors

Secreted factors such as FGFs, Wnts and BDNF are all recognized as robust synaptogenic factors and functional blockade though pharmacological antagonists or mutation experiments causes significant loss of synapse number during development (see Chapters 1.3.1 and Chapter 1.3.2). Secreted synaptogenic factors regulate the clustering of pre- and postsynaptic molecules during embryonic and early postnatal development (McAllister, 2007; Waites et al., 2005). FGFs, Wnts and BDNF are all expressed in the adult brain; however their function within the mature central nervous system is not fully understood. In the adult brain FGF signaling regulates neurogenesis (Palmer et al., 1995; Zhao et al., 2007) and synaptic plasticity (D'Sa et al., 2007; Zhao et al., 2007), but FGFs have not been reported to regulate or participate in synapse stability or maintenance. A potential role for Wnt-mediated synaptic maintenance was inferred from a recent study investigating Wnt activity in adult brain function (Gogolla et al., 2009) and further investigations are required to validate this role. In contrast, BDNF is the only secreted factor to have been directly investigated as a synaptic maintenance factor in addition to its roles in synapse assembly, maturation and function (Hu et al., 2005). Here I will focus on BDNF and Wnt function and describe their potential as maintenance factors at central synapses.

BDNF is expressed in the adult brain (Yang et al., 2009b) and is regulated by activity-dependent mechanisms (Genoud et al., 2004; Yoshii and Constantine-Paton, 2010). In the developing brain, BDNF enhances synaptogenesis, synaptic transmission and mediates activity-dependent synapse maturation and plasticity (Figurov et al., 1996; Genoud et al., 2004; Lin and Koleske, 2010; Patterson et al., 1996; Yoshii and Constantine-Paton, 2010) (see also Chapter 1.3.1). During *Xenopus* development, blockade of endogenous BDNF using functional antibodies induces retinal axon branch pruning and disassembly of Synaptobrevin clusters (Hu et al., 2005). The number of GFP-synaptobrevin axonal clusters was reduced within 2-hours of anti-BDNF injection and became significant at 4-hours, and by 8-hours post injection ~50% of GFP-synaptobrevin clusters were lost (Hu et al., 2005). Interestingly, Hu *et al.* (2005) discovered that only a subset of Synaptobrevin clusters were preferentially stabilized and maintained by BDNF signaling; those that were initially assembled in a BDNF-dependent manner. Synaptobrevin clusters that formed during BDNF-blockade remained stable and resilient to reduced levels of endogenous BDNF (Hu et al., 2005).

Studies of the barrel cortex reveal that adult *BDNF* heterozygous mice display normal distribution of synapses but the assembly of new synapses during whisker stimulation is inhibited (Genoud et al., 2004). This suggests that *in-vivo,* existing synapses continue to be maintained when BDNF expression is reduced, but synapse assembly is restricted (Genoud et al., 2004). Adult *BDNF* heterozygous mice display synaptic deficits in the hippocampus such as reduced number of docked vesicles in CA1 synapses and impaired LTP (Korte et al., 1995; Patterson et al., 1996; Pozzo-Miller et al., 1999). BDNF mediated synapse function and plasticity is regulated by BDNF induced local translation and transcription (Santos et al., 2010),

but whether the proteins synthesized under BDNF control also participate in synaptic maintenance has yet to be revealed. Whilst these studies show a role for BDNF in the adult brain for synapse assembly and function, it is not clear whether BDNF signaling in the mature brain regulates synapse stability as suggested by developmental studies in *Xenopus* (Hu et al., 2005).

Wnts are expressed in the adult brain (Gogolla et al., 2009; Shimogori et al., 2004) and are regulated by neuronal activity (Ataman et al., 2008; Chen et al., 2006; Gogolla et al., 2009; Wayman et al., 2006; Yu and Malenka, 2003). *In-vivo* studies of Wnt activity in the adult hippocampus reveal Wnt7a/b is expressed in an activity-dependent manner and is required to stimulate the formation of new synapses in response to environmental enrichment (EE) (Gogolla et al., 2009). Wnt blockade by localized microinjection of the antagonist Sfrp1 prevents the formation of new synapses following EE suggesting that Wnt signaling, like BDNF (Genoud et al., 2004), regulates synaptogenesis in the adult brain (Gogolla et al., 2009). Importantly, the authors report when the mice were returned to normal housing conditions following EE, synapse numbers returned to control levels over the following 8-10 weeks, this progressive loss of synapses was concomitant with reduced Wnt7a/b expression suggesting that sustained Wnt signaling is required to maintain the newly formed synapses (Gogolla et al., 2009). However, a role for Wnt mediated synaptic maintenance cannot be directly inferred from this study as Wnt signaling also regulates synapse plasticity (Chen et al., 2006; Lim et al., 2010) and maturation (Varela-Nallar et al., 2010), and therefore deficits in maturation may account for the synapse loss observed in this study. Whilst BDNF and Wnt proteins may be candidates for regulating synaptic maintenance, their roles in the adult brain with regard to maintaining synapses remains poorly understood.

The cytoskeleton

- *Microtubules*

Microtubules exist in both stable and dynamic pools and their organization regulates cytoplasm structure and stability. Dynactin is a multi-subunit protein complex that regulates microtubule organization, stability and dynamics through interactions with plus-end microtubule binding proteins, which capture and anchor microtubules at the cell cortex (Quintyne et al., 1999; Schuyler and Pellman, 2001). Dynactin also regulates axonal transport of organelles along microtubules (Ahmad et al., 1998; Burkhardt et al., 1997; Waterman-Storer et al., 1997), and the localization of molecules and organelles required for regulating microtubule dynamics and availability of components to the presynaptic terminal (Eaton et al., 2002). Dynactin binds with Tau proteins, which are soluble microtubule associated proteins that act as molecular linkers between Dynactin and Tubulin heterodymers (Magnani et al., 2007). This complex modulates microtubule stability and axonal transport of organelles and other molecular cargos (Magnani et al., 2007). Evidence suggests that Tau mutations, which are associated with a number of neurodegenerative diseases including Alzheimer's disease, fronto-termporal dementia and

parkinsonism linked to chromosome-17 (Selkoe and Podlisny, 2002; Yancopoulou and Spillantini, 2003) affect Dynactin distribution and association with microtubules with deleterious effects on axonal stability and transport, which in turn contribute to the neurodegeneration that hallmarks these diseases (Magnani et al., 2007).

At central synapses Dynactin binds with the cytoskeletal scaffold protein Spectrin, which forms a network on the intracellular side of the plasma membrane and maintains plasma membrane integrity (Holleran et al., 2001). Thus, Dynactin links microtubules with the plasma membrane, which together confer structural support to the synapse. At the *Drosophila* NMJ, disruption of the Dynactin complex by RNAi or mutation severely impairs presynaptic stability and function by inducing presynaptic retraction (Eaton et al., 2002). Immunocytochemical, ultrastructural and electrophysiological studies have revealed that presynaptic Spectrin and the adapter protein Ankyrin2 are essential for synapse stability at the *Drosophila* NMJ by regulating microtubule and CAM organization (Koch et al., 2008; Pielage et al., 2008; Pielage et al., 2005). Ankyrin proteins are adapter proteins that link the spectrin-based membrane skeleton with specific transmembrane proteins such as L1CAM, NrCAM, voltage-gated sodium channels and potassium channels, and stabilize their positions within specific membrane domains (Jenkins and Bennett, 2001; Pan et al., 2006; Yang et al., 2007; Zhou et al., 1998). A giant isoform of Ankyrin2 (Ank-2L) has been identified as a core mediator of presynaptic stability at the *Drosophila* NMJ (Koch et al., 2008; Pielage et al., 2008). Spectrin and Ank-2L mutants are phenotypically similar (Koch et al., 2008; Pielage et al., 2008; Pielage et al., 2005); loss of presynaptic Spectrin induces loss of Synaptotagmin and the CAMs Neuroligan and Fascilin II (Pielage et al., 2005), and Ank-2L mutants display loss of Fascillin II and active zone markers (Koch et al., 2008; Pielage et al., 2008). Importantly, both mutant strains display severe disruption of microtubule organization and stability, and retraction of presynaptic terminals from postsynaptic specializations (Koch et al., 2008; Pielage et al., 2008; Pielage et al., 2005). Furthermore, the Ank-2L phenotype is dose-dependent (Koch et al., 2008). Mutation analyses suggest Ank-2L functions downstream of Spectrin to stabilize microtubules and *in-vitro* analysis has identified the Ank-2L C-terminal domain that binds directly to microtubules to regulate their organization and stability (Pielage et al., 2008). The evidence suggests that Spectrin, via Ank-2L interactions, provide a stabilizing link between CAMs and the microtubule cytoskeleton. In addition, the authors propose that disrupted microtubule organization impedes transport of trophic factors that contribute to maintaining synaptic function and stability (Pielage et al., 2005). Importantly, Ankyrin dysfunction has been implicated with vertebrate neurodegeneration, which reveals a conserved role for Ankyrin in maintaining synaptic stability and integrity (Scotland et al., 1998; Zhou et al., 1998).

- *Actin*

As previously described, actin plays a major role in regulating pre- and postsynaptic function and synapse morphology (see Chapters 1.2.1 and 1.2.2). Critically, the actin cytoskeleton provides a dynamic platform that regulates the equilibrium between synapse assembly and

disassembly, and stability and plasticity through tightly regulated cycles of polymerization and depolymerization (Cingolani and Goda, 2008; Dillon and Goda, 2005). Analyses of fluorescent recovery after bleaching (FRAP) experiments show that molecular components of the PSD including PSD95, PSD-Zip45 (Homer 1c), GKAP and Shank undergo a steady state exchange between a stable pool and a dynamic fraction with differential dynamics (Kuriu et al., 2006). Time-lapse studies of cultured hippocampal neurons demonstrate that disruption of actin polymerization by latrunculin-A induces a rapid loss of the scaffolding proteins PSD-Zip45, GKAP and Shank from the PSD (Kuriu et al., 2006). Actin depolymerization specifically induces disassembly of the dynamic fraction of PSD-Zip45, GKAP and Shank; in contrast PSD-95 is relatively resistant to changes in actin dynamics (Kuriu et al., 2006). In addition, immunoprecipitation assays reveal that actin depolymerization impairs interactions between the PSD scaffolding proteins PSD-Zip45 and Shank (Kuriu et al., 2006). Together, these data suggest that F-actin regulates the integration and maintenance of multiple, yet specific, scaffolding proteins within the PSD under basal conditions. Chemical enhancement of synaptic transmission with bicucullin induces rapid remodeling of F-actin at postsynaptic sites (Kuriu et al., 2006; Okabe et al., 2001b). Bicucullin rapidly redistributes PSD-Zip45 and Shank within the PSD and also alters the binding affinity between different postsynaptic scaffolding proteins; this activity-dependent dynamic behavior is suppressed by latrunculine-A (Kuriu et al., 2006). Based on these findings, a model has been presented whereby the cytoplasmic side of the PSD interacts with F-actin, which is subject to dynamic remodeling by polymerization and depolymerization by activity states (Kuriu et al., 2006). In turn these changes modulate the localization specific PSD scaffolding proteins to remodel and maintain its composition and integrity (Kuriu et al., 2006).

Transcription, translation and protein degradation

Activity-dependent transcription and local translation have been extensively investigated over the past decade primarily in the context of synaptic plasticity and the maintenance of LTP. However, synaptic plasticity and LTP lie outside the scope of this thesis and as such will not be discussed. The focus of this discussion is the role of transcription, local translation and protein degradation in maintaining synapse structure. As previously described, protein composition at synaptic sites is highly dynamic with many proteins displaying short half-lives and rapid turnover. Whilst protein trafficking replenishes proteins at synapses to ensure sustained synaptic integrity, synapses still require the *de novo* formation of proteins to balance losses due to protein degradation.

- *Transcription*

Postsynaptic calcium influx activates calcium/calmodulin-dependent protein kinase II (CaMKII), which in addition stimulating local signaling cascades, initiates signaling to the nucleus to activate the cAMP response element binding protein (CREB) transcription factor and induce

gene expression (Cohen and Greenberg, 2008; Lonze and Ginty, 2002; Redmond et al., 2002; Wayman et al., 2006). Transcriptional targets of CREB include the synaptic proteins Synaptotagmin, VAMP2, SV2, Syntaxin, SNAP, Shank and EphrinA5 (Impey et al., 2004). In addition to the replenishment of synaptic proteins, activity dependent transcription via CREB is necessary to supply the neurotrophic factors BDNF and Wnt2 (Ghosh et al., 1994; Tao et al., 1998; Wayman et al., 2006; West et al., 2001). Expression and subsequent secretion of Wnt2 is required developmentally for dendritic arborization (Wayman et al., 2006). BDNF transcription and secretion enhances synaptic transmission by stimulating local protein translation (Kang and Schuman, 1996), and mediates activity-dependent synapse maturation and the maintenance of synaptic circuits (Genoud et al., 2004; Yoshii and Constantine-Paton, 2010) (see also Chapter 1.3.1 of this thesis).

- *Local translation*

Synapse-associated polyribosome complexes (SPRCs) where first identified almost 30 years ago (Steward and Levy, 1982). SPRCs selectively localize to membranous cisterns below postsynaptic sites within the dendrite, often at the very base of the spine (Steward, 1983a; Steward and Fass, 1983b; Steward and Levy, 1982). A rapid translational response can occur within 5 minutes of postsynaptic stimulation (Weiler et al., 1997) and is locally mediated by calcium dependent mRNA translation within the dendrite or spine (Aakalu et al., 2001; Kindler et al., 2005; Wu and Cline, 1998). This ensures sufficient synaptic proteins are available to maintain function (Steward and Schuman, 2001) and importantly, enables individual synapses to control their strength by providing a mechanism for localized response to stimulation.

A number of mRNAs have been identified *in-vivo* by in-situ hybridization of hippocampal and cerebellar slices, and include the signaling molecules CaMKII, Calmodulin, and the inositol triphosphate receptor; cytoskeletal associated proteins Neurofilament protein-68, MAP2 and Arc; and membrane associated proteins such as NMDA-R1, Glycine receptor alpha subunit and G-protein gamma subunit (Steward and Schuman, 2001). Different neuronal subtypes differentially express different mRNAs temporally and spatially, which suggests that local protein translation is determined by requirements of the cell, and furthermore, within discrete regions of the dendritic arbor (Bian et al., 1996; Steward and Schuman, 2001; Steward et al., 1998). The above analyses were performed on acute slices that were not subjected to experimental stimulation, which suggests that dendritic mRNAs and SPRCs are present under basal conditions. This suggests either that local translation occurs constitutively to replenish and maintain synaptic protein composition or, that the translation machinery is present and waiting for activation by postsynaptic stimulation to facilitate the molecular and structural modifications required for synaptic plasticity. Alternatively, both of these functions may be met by local protein translation.

In the axon, local translation plays a critical role in axon guidance during development (Lin and Holt, 2008; Piper and Holt, 2004). However, whether local translation is a widespread

mechanism for regulating presynaptic structure and/or function in mature axons is not clear, as there is little direct evidence for axonal polyribosomes (Giuditta et al., 2002; Piper and Holt, 2004). A number of mitochondrial associated protein mRNAs have been identified in synaptosomal preparations from the squid giant axon (Giuditta et al., 2002). This indicates that mitochondrial function may be regulated by local mechanisms and suggests a relationship between presynaptic function and local energy supply (Giuditta et al., 2002). In addition, mRNAs that encode neurofilament proteins have been identified in synaptosomes of the squid brain (Crispino et al., 1993). Whilst presynaptic mRNA for mitochondrial associated proteins and cytoskeleton components suggests local translation of proteins that would support synaptic maintenance, further research is necessary to determine if these mRNAs are indeed translated, and if so, what role do they play at the synapse.

Two studies have identified local translation in presynaptic terminals of mature synapses (Wang et al., 2009; Yan et al., 2009). In *Aplasia,* activation of postsynaptic calcium signaling is required for 5HT-induced translation of Sensorin in the presynaptic terminal (Wang et al., 2009). Sensorin mRNA localizes to a subset of presynaptic terminals and is required for 5HT-induced long-term fascilitation of the gill-withdrawal reflex (Wang et al., 2009). In mature *C. elegan* neurons, stability of axonal mRNA that encodes the transcription factor CEBP1 (CCAAT/enhancer-binding proteins) is necessary for maintaining presynaptic morphology (Yan et al., 2009). Both cepb1-mRNA and CEBP1 protein are present in presynaptic motoneuron terminals suggesting local mRNA translation (Yan et al., 2009). Importantly, inappropriate upregulation of cebp1 mRNA by conditional overexpression in mature neurons significantly reduces the number of synaptic sites, and remaining synapses display abnormal morphologies compared to controls (Yan et al., 2009). How CEBP1 regulates synaptic maintenance is not addressed in this report, and whether it is transported to the nucleus to function as a transcription factor remains unclear.

Due to the immense scientific interest in how the CNS is modified by experience and the role of activity-dependent mechanisms in structuring the brain, little attention has been given to the role of protein synthesis in maintaining synapses under basal conditions. Whilst it may be reasonable to speculate that *de novo* proteins are incorporated into existing stable synaptic sites to maintain function and structure, further research is required to identify and characterize mechanisms that may stimulate protein production within the parameters of normal synaptic function.

- *Protein degradation*

A role for activity-dependent protein degradation has been demonstrated as an important mechanism for regulating synaptic protein turnover within the PSD, and therefore contributes to maintaining correct protein composition at postsynaptic sites (Ehlers, 2003). Pulse-chase analyses of radioactively labeled cysteine/methionine reveals that elevated synaptic activity increases the turnover rate of many PSD proteins in mature synaptosomal preparations by

stimulating ubiquitin conjugation of specific PSD proteins (Ehlers, 2003). Conversely, blockade of synaptic activity with TTX reduces the turnover rate and leads to the eventual loss of proteins such as of NR2A, mGlu-R1α, PSD95, Homer, MyosinVa, CaMKII and PKCγ (Ehlers, 2003). These data suggest that activity blockade impedes the turnover of PSD proteins and inhibits the dynamic exchange of PSD constituents.

Summary

Electromicroscopy and long-term confocal microscopy imaging studies of mature rodent brains reveal that the majority axo-dedritic contacts are stable (Arellano et al., 2007; Harris, 1999). In contrast, the molecular composition of synapses is highly dynamic, whereby synaptic proteins are degraded and shuttle in and out of synapses at a remarkable rate (Ehlers et al., 2007; Gray et al., 2006; Kielland et al., 2009) by lateral diffusion and exo- and endocytosis (Bredt and Nicoll, 2003; Cognet et al., 2006; Collingridge et al., 2004; Malinow and Malenka, 2002; Wenthold et al., 2003).

Synaptic activity is the key stimulus for maintaining the molecular composition of synapses. Studies demonstrate that synaptic proteins and signaling molecules are synthesized in an activity dependent manner (Steward and Schuman, 2001; West et al., 2001). Furthermore, activity-dependent mechanisms regulate the trafficking of synaptic proteins to active synaptic sites (Ehlers et al., 2007; Kielland et al., 2009). Critically, synaptic transmission organizes and maintains the presynaptic active zone architecture (Bouwman et al., 2004), postsynaptic receptors (Ehlers et al., 2007; Kielland et al., 2009; Ripley et al., 2010) and adhesion molecules (Chen et al., 2010). CAMs subsequently regulate the cytoskeleton (Bamji, 2005; Chen et al., 2010; Rimm et al., 1995), which feeds-back to the PSD to regulate its molecular composition possibly by providing a supportive framework and affecting PSD protein-protein interactions (Kuriu et al., 2006). Presynaptically the actin cytoskeleton organizes SV clusters and recycling (refer to Chapter 1.2.1.3 for a detailed discussion) to ensure sustained presynaptic function. In addition to organizing these functionally diverse proteins, analyses reveal that activity stimulates complex interactions between them, which adds further structure to support synapse stability and maintenance.

Taken together, the data supports a model for dynamic synapse behavior induced by activity driven mechanisms (Bhatt et al., 2009; Bourne and Harris, 2008; Renner et al., 2008) and demonstrates that activity regulates and maintains synaptic composition. Whilst secreted factors such as BDNF and Wnts are expressed and released by activity-dependent mechanisms, their role in regulating synaptic maintenance remains poorly defined.

1.4. Wnt signaling

The term Wnt is an amalgam of the *Drosophila wingless* (*wg*) gene and the vertebrate oncogene *int-1* (Nusse et al., 1991). Wnt proteins are a family of highly conserved secreted

glycoproteins that activate a number of diverse intracellular signaling pathways (Gordon and Nusse, 2006; Logan and Nusse, 2004; van Amerongen and Nusse, 2009). Wnts regulate critical events such as placenta development and early embryonic patterning; cell fate and cell proliferation; neuronal migration, axon guidance and synaptogenesis (Logan and Nusse, 2004; Salinas and Zou, 2008; Speese and Budnik, 2007). Subsequently, regulated Wnt signaling is required for ongoing cellular and tissue homeostasis, and the consequences of aberrant Wnt signaling are well established in cancer biology (Clevers, 2006; Klaus and Birchmeier, 2008; Logan and Nusse, 2004). The array of diverse cellular outcomes (see Table 1) is achieved through multiple Wnt ligands interacting with numerous receptors and co-receptors that initiate distinct signaling events both locally and via the nucleus to elicit transcription of target genes (Kikuchi et al., 2007; Mikels and Nusse, 2006; van Amerongen and Nusse, 2009). Additional levels of complexity within Wnt signaling are conferred by temporal and spatial expression of secreted regulatory factors that antagonize or activate specific Wnt pathways, or act as a switch between different Wnt pathways.

The *wingless* gene and its role in establishing segment polarity were identified 35 years ago through analyses of mutant embryos (Sharma and Chopra, 1976). Insightfully, the authors postulated that *wingless* might be involved in specifying embryonic cell fate; 10 years later molecular analyses and antisense probes fully defined the *wingless* gene and its 3kb transcript and provided compelling evidence for its role in early *Drosophila* embryonic segmentation and patterning (Baker, 1987; Cabrera et al., 1987). Conversely, *Int-1* was characterized as an oncogene involved in viral mammary tumorgenesis in mice in the early 1980s (Nusse et al., 1991). By the mid 1980s analysis of the structure and nucleotide sequence of int-1 predicted the gene product was a secreted protein, but its function in tumorgenesis and mechanism of action was unknown (van Ooyen and Nusse, 1984). The important finding that these two genes, *wingless* and *int-1*, were in fact orthologs was reported a few years later when genomic and cDNA clones of the Drosophila ortholog to *int-1* (*Dint1*) were isolated and found to be identical and map to the same position as *wingless* (Rijsewijk et al., 1987).

Traditionally, the downstream signaling cascades initiated by Wnt ligands have been broadly categorized as canonical-Wnt and non-canonical-Wnt. Most Wnt signaling pathways are activated by Wnt ligands binding with Frizzled (Fz) receptors at the plasma membrane, which activates the cytoplasmic scaffolding protein Disheveled (Dvl); it is at this point that Wnt signaling diverges into different pathways. Canonical Wnt signaling refers to the most characterized pathway that involves stabilization of cytosolic β-catenin, its translocation to the nucleus and activation of β-catenin responsive target genes via TCL/LEF (Tcell factor/lymphoid enhancer factor transcription factors). Non-canonical, or β-catenin independent, pathways encompass a number of different signaling cascades, the most well known being the planar cell polarity (PCP) pathway and the calcium-Wnt pathway. Both PCP and calcium-Wnt pathway affect local changes in the cytoskeleton (Angers and Moon, 2009; Gordon and Nusse, 2006; Kikuchi et al., 2007; van Amerongen and Nusse, 2009) (Figure 1.4). Which signaling pathway is

ultimately activated is determined at the plasma membrane by specific receptors or combination of receptors.

Wnt isoform	Stem cell control	Cell proliferation	Cell differentiation	Gastrulation	Axis formation	Cell migration	Organogenesis	Neurogenesis	Axon guidance	Axon remodelling	Dendritogenesis	Synaptogenesis	Synapse function	Cancer	Neurologic diseases	Other diseases
Wnt1	✓	✓	✓						✓			✓	✓	✓		
Wnt2							✓				✓			✓	✓	
Wnt2b/13							✓									
Wnt3	✓			✓	✓			✓		✓		✓		✓		✓
Wnt3a		✓	✓					✓		✓		✓	✓		✓	
Wnt4							✓		✓					✓		✓
Wnt5a			✓		✓	✓	✓	✓	✓			✓		✓		
Wnt5b																✓
Wnt6														✓		
Wnt7a			✓							✓		✓	✓			✓
Wnt7b							✓				✓	✓		✓		
Wnt8a																
Wnt8b														✓	✓	
Wnt9a														✓		
Wnt9b																
Wnt10a														✓		✓
Wnt10b														✓		✓
Wnt11					✓		✓					✓				
Wnt16			✓													

Table 1. Comparison of function of different Wnt isoforms. The summary table highlights the diversity of function between the 19 known Wnt isoforms in human and mouse. Whilst Wnt signalling has been implicated in a number of neurogenic diseases, roles for specific Wnt isoforms have yet be characterized. The absence of a tick does not necessarily preclude a role for isoforms in specific functions; rather, the Wnt isoform may not have not been investigated in the given cellular context(s). As described in the main text, cellular outcomes in response to Wnt ligands are critically determined by temporal-spatial expression and availability of components of Wnt signalling pathways. The table summarizes evidence primarily from vertebrate studies using both gain and/or loss of function studies (Budnik and Salinas, 2011; Ciani and Salinas, 2005; De Ferrari and Moon, 2006; Fuerer et al., 2008; Gordon and Nusse, 2006; Inestrosa and Arenas, 2010; Logan and Nusse, 2004; Salinas and Zou, 2008; van Amerongen and Nusse, 2009).

1.4.1. The Wnt family, post-translational modifications and secretion

The mouse and human genomes contain nineteen different Wnt isoforms (for detailed list see The Wnt Homepage at www.stanford.edu/group/nusselab/cgi-bin/wnt/); this diversity is likely to reflect tissue specific expression, rather than specification of cellular outcome. For example,

Wnt5b and Wnt11 both activate non-canonical Wnt signaling in developmental models, yet in C57MG breast cells both Wnts weakly activate canonical signaling (Chien et al., 2009). Therefore, downstream signaling depends on the cellular context and is most likely determined by the availability of receptor(s) and downstream signaling molecules.

Studies have found that Wnt proteins are subject to substantial post-translational modifications including glycosylation and palmitoylation that are necessary for secretion and receptor binding, and may also confer functional differences between the different Wnt isoforms (Komekado et al., 2007; Kurayoshi et al., 2007; Takada et al., 2006). For example, glycosylation of murine Wnt3a at conserved cystein residues is necessary for subsequent palmitoylation at serine residues, which in turn is required for intracellular transport of Wnt3a from the endoplasmic reticulum (ER) (Takada et al., 2006). Mutant mice or *Xenopus* embryos defective in Ser209 palmitoylation accumulate Wnt3a in the ER and exhibit failures in Wnt secretion, a phenotype that is mimicked by mutations in *porcupine*, which expresses an acyltransferase protein required for Ser209-dependent palmitoylation (Komekado et al., 2007; Takada et al., 2006; Zhai et al., 2004). Furthermore, phenotypic analyses of mutant mice reveal that palmitoylation of Wnt5a at Cys104 regulates binding with its cognate Fz receptor and is required to trigger intracellular signaling (Kurayoshi et al., 2007). Acylation of Wnts with glycosyl groups and/or palmitate facilitates Wnt binding with ECM molecules such as heperan sulphate proteoglycans, which may be functionally important in establishing the Wnt morphogen gradients that regulate embryonic patterning (Bartscherer and Boutros, 2008; Bradley and Brown, 1990; Reichsman et al., 1996). Therefore, extensive post-translational modifications of Wnt proteins are critical for their function. However, these modifications make purification and commercial production of active recombinant Wnt proteins notoriously difficult and the production of Wnts with variable activity, which has hindered Wnt research considerably.

Intracellular transport of modified Wnts from the Golgi apparatus to the cell surface for secretion is regulated by Evenness interrupted (Evi), which is also known as Wntless (Wls) (Banziger et al., 2006; Bartscherer et al., 2006; Korkut et al., 2009). Evi is a multispan transmembrane protein that localizes with known components of the secretory pathway including the Golgi, endocytic vesicles and the plasma membrane (Bartscherer et al., 2006). *Drosophila* Evi mutants accumulate Wg in the Golgi and phenocopy *wg* mutants (Banziger et al., 2006; Bartscherer et al., 2006; Korkut et al., 2009; Port et al., 2008). In addition, colocalization and coimmunprecipitation experiments reveal *in-vitro* interactions between Evi and Wg (Banziger et al., 2006). These data suggest that Evi and Wnt/Wg associate at the Golgi, whereby Wnt/Wg is transported within vesicles and Evi becomes a cargo receptor facilitating Wnt/Wg transport to the plasma membrane (Bartscherer and Boutros, 2008). Furthermore, Evi regulates Wnt/Wg release and diffusion across the *Drosophila* NMJ via exosome-like vesicles that are secreted from the synaptic terminal (Korkut et al., 2009).

1.4.2.Wnt signaling at the receptor level

Frizzled receptors

Ten mammalian Fz isoforms have been identified all of which share common structural motifs; a cysteine rich domain (CRD) at the extracellular N-terminal followed by an amino acid sequence that hydropathy analyses predict as a seven-pass transmembrane structure (Schulte and Bryja, 2007). The first Wnt receptor identified was a member of the Drosophila Frizzled family (DFz) (Bhanot et al., 1996). Using multiple *in-vivo* strategies the authors demonstrated over-expression of DFz2, stimulated with Wg increased armadillo levels (the β-catenin homologue) indicating activation of the Wnt-canonical pathway. Bhanot *et al* (1996) identified the extracellular CRD of DFz2 as the Wg binding site, and the PDZ binding domain as a binding site for the cytoplasmic scaffold protein Disheveled (Dvl). Importantly, the Fz-PDZ binding domain is required for initiating intracellular signaling cascades (Cadigan et al., 1998) via interactions with Dvl, and also through trimeric G-proteins (Cadigan and Liu, 2006; Schulte and Bryja, 2007).

The crystal structure of Fzs predicts that Fzs also interact with each other through CRD/CRD interactions (Dann et al., 2001). Fz receptor dimerization or oligomerization is supported by in-vivo studies that suggest oligomerization regulates receptor maturation and presentation to the cell membrane (Kaykas et al., 2004) and occurs in a Wnt-dependent manner (Dann et al., 2001).

Studies suggest that specific Fz isoforms can trigger specific signaling pathways. For example, Wnt5a binds with endogenous Fz to inhibit canonical-Wnt signaling in cultured hippocampal neurons (Davis et al., 2008; Liu et al., 2005a); however, when exogenous Wnt5a is applied to cells overexpressing Fz5, or Fz4, then Wnt5a stimulates canonical-Wnt signaling (He et al., 1997; Mikels and Nusse, 2006). This study suggests that the receptor type determines downstream signaling events. Furthermore in *Drosophila*, DFz1 mutants display deficits in Wnt PCP signaling, whereas DFz2 mutants do not (Kikuchi et al., 2007). Whilst the mechanisms of Fz signaling and activation of downstream molecular intermediates remain poorly understood evidence supports a role for trimeric G proteins (Kikuchi et al., 2007).

The primary endogenous Fz ligands are Wnt proteins. However the specificity of different Wnts for binding with specific Fz isoforms remains poorly understood as ligand receptor binding assays have been impeded due to problems in purifying Wnts in an active form. In addition to Wnts, Fzs also interact with secreted Frizzled related proteins (Sfrps), Norrin and R-spondin. Norrin and R-spondin are broadly classified as non-conventional activators of the canonical-Wnt pathway as they result in elevated levels of stable β-catenin and subsequent gene expression through TCF/LEF (Nam et al., 2006; Schulte and Bryja, 2007; Xu et al., 2004). These proteins are active in myogenesis and ocular development respectively (Kikuchi et al., 2007).

LRP5/6

LRP-5 and -6 are highly homologous single pass transmembrane proteins that are generally involved in ligand endocytosis, signaling and metabolism (He et al., 2004). Loss of function studies for LRP5/6 and the fly homologue, Arrow, have provided compelling evidence for their role in positively regulating canonical-Wnt signaling (He et al., 2004). In both fly and mouse models, abrogation of this single-pass transmembrane protein phenocopies canonical-Wnt mutations (Cadigan and Liu, 2006; He et al., 2004; Pinson et al., 2000), yet no deleterious effects are found in *arrow* mutants in PCP function (Wehrli et al., 2000). Furthermore, overexpression of LRP6 that is truncated at the intracellular domain acts as a dominant-negative to inhibit canonical Wnt signaling in Xenopus embryos and conversely, overexpression of LRP6 that is truncated at the extracellular domain acts as a constitutively active agonist of canonical Wnt signaling (Mao et al., 2001; Tamai et al., 2000). Wnt signaling rapidly induces (~15 minutes) the formation of large membrane-associated LRP6 aggregates in a Dvl dependent manner that colocalize with other components of canonical Wnt signaling including Fz8, Dvl2, GSK3β and Axin (Bilic et al., 2007). A model for Wnt signaling by formation of an "LRP6-signalsome" has been suggested whereby a high concentration of LRP6 is stabilized by a scaffold platform of polymerized Dvl, which initiates CK1 phosphorylation and Axin recruitment (Schwarz-Romond et al., 2007a; Schwarz-Romond et al., 2007b).

Ryk and Ror

Whilst Fz and LRP5/6 mediate canonical Wnt signaling via β-catenin, the atypical tyrosine kinase receptors Ryk and Ror are generally characterized in non-canonical Wnt signaling. Ryk and ROR activate molecular intermediates such as heterotrimeric G-proteins, intracellular calcium, small GTPases, the cytoskeleton and the JNK and MAPK pathways to regulate cellular polarity, motility, migration and axon guidance (Cheyette, 2004). Mutation experiments demonstrate a functional requirement for Ryk and Derailed (the Ryk *Drosophila* homologue) in Wnt5-mediated axon guidance (Lu et al., 2004; Yoshikawa et al., 2003). Importantly, Derailed functions in the absence of Fz receptors (Yoshikawa et al., 2003) by signaling through Src tyrosine kinases (Wouda et al., 2008). Although Ryk is generally characterized as a non-canonical Wnt agonist, cell-culture systems reveal that Ryk also forms complexes with Fz to activate canonical-Wnt signaling (Cheyette, 2004) via TCF-mediated transcription (Lu et al., 2004).

Ror proteins are single pass transmembrane receptors that contain an extracellular CRD and Fz domain, and an intracellular tyrosine kinase domain (Green et al., 2008; Oishi et al., 2003; Xu and Nusse, 1998). Studies from *Ror2* mutant mice demonstrate that Ror proteins are required for signaling through the PCP pathway alongside the conserved components Fz, Dvl, Van Gogh and Flamingo (Yamamoto et al., 2008). This is consistent with in-vitro binding assays that reveal Ror2 binds with Fz2 to activate the non-canonical JNK pathway (Oishi et al., 2003). Ror2 can

also potentiate TCF-mediated gene expression (Billiard et al., 2005; Li et al., 2008). However, evidence from *C. elegans* and cell culture systems demonstrate that Ror proteins can bind with Wnts to block β-catenin stabilization, and therefore canonical-Wnt signaling (Billiard et al., 2005; Green et al., 2007).

1.4.3.Wnt signaling pathways

The canonical-Wnt pathway

The canonical-Wnt pathway is a complex signaling cascade that ultimately elicits a transcriptional response via β-catenin binding with specific transcription factors (Figure 1.4). The array of canonical-Wnt target genes is substantial and diverse (see the Wnt Homepage http://www.stanford.edu/group/nusselab/cgi-bin/wnt/main for a comprehensive list). Many Wnt target genes can be broadly categorized as participating in cell differentiation, cell proliferation, cell adhesion and regulation of Wnt signalling. Canonical-Wnt signalling promotes transcription of the early response transcription factors *c-jun*, *fra1* and *uPAR,* which together comprise the heterdimeric transcription factor activator protein 1 (AP1) (Mann et al., 1999). AP1 target genes in turn regulate cellular processes such as differentiation, proliferation, migration and apoptosis by targeting genes that regulate chromatin remodelling, the cytoskeleton, glycoproteins within the extracellular matrix (Ozanne et al., 2007) and cell-adhesion molecules including *Nr-CAM* (neuronal cell adhesion molecule) (Conacci-Sorrell et al., 2002), *fibronectin*, *CD44* and *Eph/ephrin* (Batlle et al., 2002; Ozanne et al., 2007). Canonical-Wnt signalling also promotes expression of *myc* genes, which function as transcription factors, transcription repressors and regulators of DNA replication (Cowling et al., 2006; Dominguez-Sola et al., 2007; Herkert and Eilers, 2010; ten Berge et al., 2008). Biological outcomes of *myc* gene expression include cell proliferation, cell growth, cell differentiation and stem cell regulation (Cowling et al., 2006; Herkert and Eilers, 2010). Canonical-Wnt signalling deploys regulatory mechanisms that activate or deactivate Wnt signaling, for example, Wnt mediated expression of *LEF1* promotes canonical-Wnt signalling in the nucleus (Hovanes et al., 2001), whereas Wnt-mediated expression of *Dkk1* and *Axin2* inhibit the pathway (Gonzalez-Sancho et al., 2005; Jho et al., 2002; Lustig et al., 2002; Niida et al., 2004; Yan et al., 2001). *Arrow/LRP* expression can be down regulated by Wnts to block signaling (Wehrli et al., 2000) and specific *Frizzleds* can be either up- or down regulated to regulate the pathway (Cadigan et al., 1998; Muller et al., 1999; Sato et al., 1999; Willert et al., 2002).

In the absence of Wnt, a macromolecular complex referred to as the “destruction complex” phosphorylates cytosolic β-catenin, which labels it for ubiquitination and proteosomal degradation, thereby inhibiting its translocation to the nucleus and the transcription of target genes (Figure 1.4). This grossly oversimplified description serves only to introduce the signaling cascade and provides a basic framework for the following discussion.

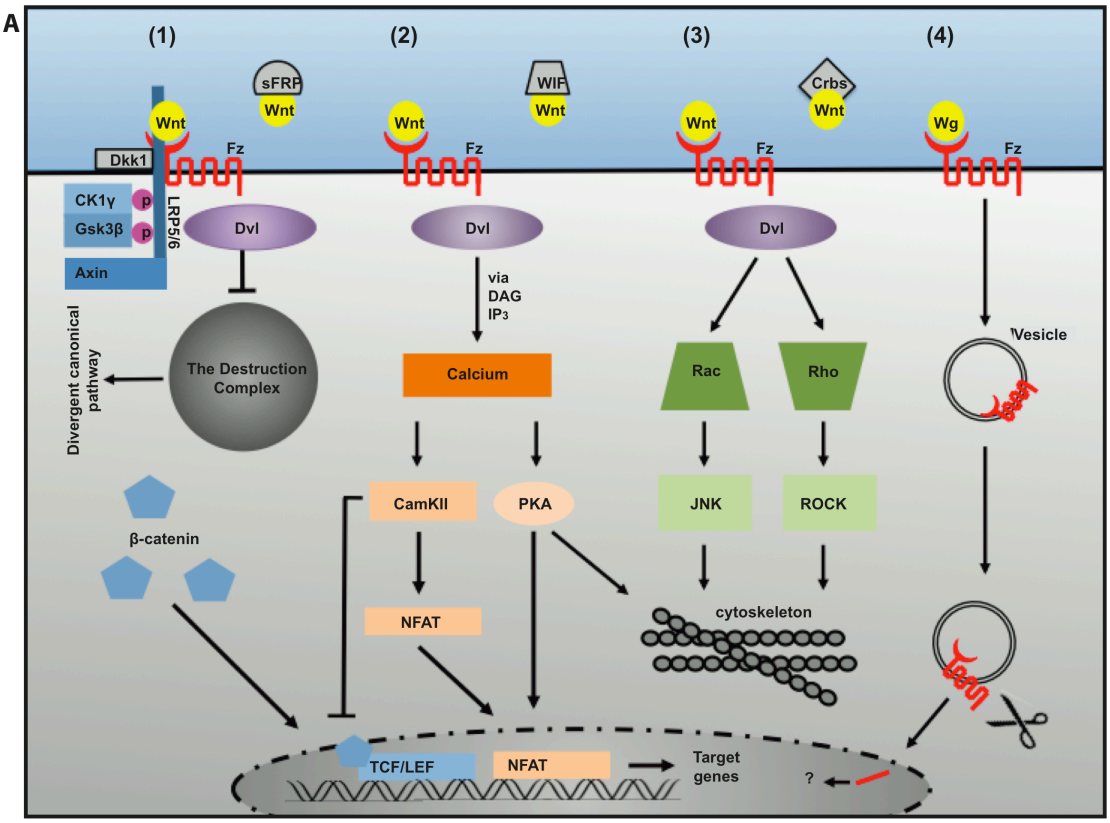
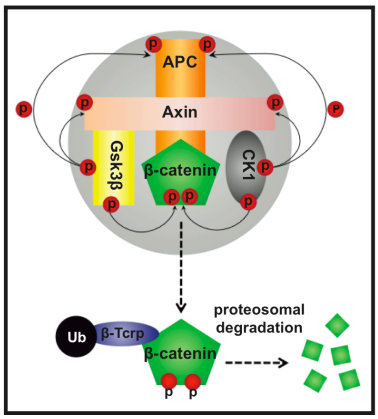


Figure 1.4. The main Wnt-signaling pathways. A) The four main Wnt signalling pathways are numbered 1-4. **(1) The canonical-Wnt pathway**: Wnt binds with Frizzled (Fz) and low-density lipoprotein receptor related (LRP5/6) to stimulate the recruitment of Disheveled (Dvl) and Axin to the plasma membrane. Casein kinase 1 (CK1) and Gsk3β are also recruited and phosphorylate LRP5/6. Formation of the Wnt/Fz/Dvl/Axin complex at the plasma membrane inhibits the destruction complex (see panel B). Stable β-catenin translocates to the nucleus to initiate transcription of target genes via TCF/LEF transcription factors. A divergent canonical-Wnt pathway bifurcates at Gsk3β.**(2) The Wnt/Ca++ pathway**: Wnt binding to Fz activates Dvl, which in turn signals through diacylglycerol (DAG) and inositol triphosphate (IP3) to release Ca++ from intracellular stores. Elevated Ca++ levels stimulate calcium/calmodulin-dependent kinase (CamKII) and protein kinase A (PKA). CamKII stimulates the transcription factor nuclear factor of activated T-cells (NFAT) and subsequent transcription of target genes. CamKII also inhibits β-catenin mediated transcription. PKA signals through Cdc42 to regulate actin dynamics. **(3) The planar cell polarity pathway** (PCP) pathway: Wnt binding to Fz activates Dvl, which in turn activates small GTPases such as Rac and Rho. Rac and Rho subsequently activate the kinases JNK and ROCK respectively, which converge on the cytoskeleton to regulate dynamics and organization. **(4) The Fz nuclear import pathway**: at the Drosophila NMJ, Fz is endocytosed from the plasma membrane following Wg/Fz binding. The N-terminal remains peri-nuclear and the C-terminal is transported into the nucleus where it associates with chromosomal regions. It is currently unknown if the C-terminal elicits a transcriptional response. Two classes of Wnt antagonists are illustrated within the figure; secreted frizzled related protein (Sfrp), Wnt inhibitory factor (WIF) and Cerebrus (Crbs) are all scavenger antagonists that sequester Wnt within the extracellular matrix and prevent Wnt binding with its cognate receptor. In contrast, Dkk1 binds to LRP5/6 at the plasma membrane and blocks receptor activation by preventing Wnt/Fz interactions.



B) The destruction complex is macromolecular complex central to canonical-Wnt signaling. Axin is the core scaffolding protein that bridges the other components. In the absence of Wnt signaling, Gskβ and CK1 phosphorylate Axin and APC, which stabilizes the complex. Gskβ and CK1 also phosphorylate the co-transcription factor β-catenin. Phosphorylation of β-catenin tags the molecule for labeling with Ubiquitin and subsequent proteosomal degradation. These constitute biochemical events ensure cytosolic β-catenin is kept to a minimum and therefore prevents β-catenin mediated transcription via TCF/LEF transcription factors.

- *Events at the plasma membrane*

As previously described, canonical-Wnt signaling is initially determined at the plasma membrane by Wnt binding to both Fz receptors and LRP5/6 co-receptors forming a hetero-oligomer at the membrane (Cong et al., 2004). Wnts trigger binding between the extracellular domains of LRP6 and the CRD of Fz (Tamai et al., 2000). The intracellular domain of LRP5/6 contains two juxtaposed phosphorylation sites that are rapidly phosphorylated (within 10-15 minutes) by GSK3β and CK1Ɣ in a Wnt dependent manner (Bilic et al., 2007; Davidson et al., 2005; Zeng et al., 2005). Fz-LRP5/6 interactions initiate two distinct events at the cell membrane, the recruitment of Dvl to Fz and the recruitment and binding of Axin to LRP5/6 (Cadigan and Liu, 2006; Cliffe et al., 2003; Mao et al., 2001).

Three highly homologous Dvl isoforms have been identified (Dvl-1, -2 and -3) and each isoform shares three conserved domains, the DIX, PDZ and DEP domains (Gao and Chen, 2010). Specification of downstream signaling depends upon which domain interacts with intracellular signaling molecules, for example the PDZ and DIX domains are required for canonical signaling and the PDZ and DEP domains are required for the PCP pathway (Axelrod et al., 1998; Boutros et al., 1998). Wnt-dependent Fz binding to Dvl induces Dvl phosphorylation (within ~20-30 minutes) and is a requirement for all Wnt signaling pathways (Gonzalez-Sancho et al., 2004) (Figure 1.4). The precise events that determine Dvl phosphorylation are not clear; candidate kinases include casein kinase1 (CK1), casein kinase2 (CK2), PAR-1, protein-kinaseC (PKC) and metastasis-associated kinase (MAK) as they have all been shown to phosphorylate Dvl, but their roles are as yet undefined (Schulte and Bryja, 2007).

Biochemical assays reveal the Dvl DIX domain is capable of reversibly polymerizing in-vitro and in-vivo, and this dynamic assemblage is important for Axin and CK1 recruitment to the plasma membrane (Schwarz-Romond et al., 2007a; Schwarz-Romond et al., 2007b). Dvl phosphorylation and polymerization is concomitant with LRP5/6 phosphorylation and aggregation and are rapid events that occur within ~15 minutes of Wnt/Fz/LRP binding (Bilic et al., 2007; Schwarz-Romond et al., 2007a; Schwarz-Romond et al., 2007b). In addition to Dvl polymerization and LRP5/6 aggregation, Fzs polymerize via CRD-CRD interactions in a Wnt-dependent manner (Dann et al., 2001). Together, these data provide evidence for a receptor aggregation model that is activated by Wnt, which serves to amplify signaling events from the plasma membrane (Huang and He, 2008).

- *Intracellular canonical-Wnt signaling*

At the center of the canonical model for Wnt signaling is a macromolecular complex referred to as the “destruction complex” (Figure 1.4 panel B). Axin is a core scaffold protein of the destruction complex (Figure 1.4 panel B) (Ikeda et al., 1998; Kishida et al., 1998; Yamamoto et al., 1999). The sequential phosphorylation of LRP5/6 by GSK3β then CK1Ɣ is understood to be

the trigger for Axin recruitment out of the “destruction complex” and affinity assays suggest that Dvl acts as a molecular chaperone to deliver Axin to the Fz/LRP complex (Cong et al., 2004). It has been suggested that Dvl-Axin binding induces conformational changes within Axin that alters its affinity for substrates of the destruction complex, which in turn lead to disassembly of the complex (Cadigan and Liu, 2006).

The core function of the destruction complex is to destabilize cytosolic β-catenin via a series of phosphorylation events (Figure 1.4 panel B). Key components of the “destruction complex” are the scaffold protein Axin, the tumor suppressor protein adenomatous polyposis coli (APC), and the kinases GSK3β and CK1 (MacDonald et al., 2009) (Figure 1.4 panel B). In the absence of Wnt, GSK3β and CK1 phosphorylate Axin and APC, which stabilizes the Axin/APC/β-catenin complex (Yamamoto et al., 1999). In addition, GSK3β and CK1 phosphorylate β-catenin (Huang and He, 2008; Yost et al., 1996); critically GSK3β phosphorylation of β-catenin depends upon stabilized Axin within the complex (Ikeda et al., 1998). Phosphorylated β-catenin is subsequently tagged by β-Trep, which is an E3 ubiquitin ligase subunit that labels β-catenin for proteosomal degradation (Kitagawa et al., 1999). These constitutive events ensure that cytosolic β-catenin levels are kept to a minimum and thus prevent β-catenin mediated transcription.

Disassembly of the destruction complex prevents phosphorylation of β-catenin and leads to the accumulation of stable β-catenin in the cytosol and it’s subsequent translocation to the nucleus (Riggleman et al., 1990). β-catenin is a transcription co-factor that interacts with TCF/LEF transcription factors to initiate target-gene transcription (Behrens et al., 1996; Molenaar et al., 1996; van de Wetering et al., 1997). As previously described canonical-Wnt target genes are both numerous and diverse, and include genes whose products are active during early embryogenesis, cancer, and regulation of Wnt signaling (Table 1).

In summary, the presence of Wnt disassembles the destruction complex by requisition of Axin, which consequently inhibits GSK3β phosphorylation of β-catenin. Stable β-catenin subsequently translocates to the nucleus to activate transcription of target genes through TCF/LEF transcription factors.

Divergent canonical Wnt pathway

The canonical-Wnt pathway has been traditionally associated with β-catenin mediated transcription of Wnt target genes. However, compelling evidence now reveals that Wnt signaling through molecular intermediates upstream of β-catenin directly stabilize microtubules through transcription independent mechanisms (Ataman et al., 2008; Purro et al., 2008; Salinas, 2007). Gsk3β phosphorylates microtubule-associated proteins such as tau, MAP1B and APC to promote dynamic behavior of the microtubule cytoskeleton (Salinas, 2007). In contrast, inhibition of Gsk3β phosphorylation promotes microtubule stability (Ciani et al., 2004; Lucas and Salinas, 1997). Endogenous Dvl1 and Axin associate with axon microtubules (Ciani et al., 2004;

Krylova et al., 2002), and ectopic Dvl1 or Axin expression prevents microtubule destabilization through Gsk3β inhibition when cells are challenged with nocodozole (Ciani et al., 2004; Krylova et al., 2002).

At the *Drosophila NMJ,* Wg activates a divergent canonical pathway in the motoneuron (Ataman et al., 2008; Franco et al., 2004; Miech et al., 2008). The MAP1B related *Drosophila* protein Futsch regulates microtubule stability and organization during synaptic growth through the Gsk3β homologue Shaggy (Franco et al., 2004; Gogel et al., 2006; Miech et al., 2008; Roos et al., 2000). Whilst the mechanisms Axin/Dvl/Gsk3β mediated microtubule stability in *Drosophila* have yet to be fully elucidated, these studies demonstrate a conserved role for Wnt signaling in regulating microtubule stability via a divergent-canonical Wnt pathway that is independent of transcription.

Evidence for a divergent-canonical pathway at vertebrate synapses has been provided by studies in mouse dorsal root ganglion neurons (Purro et al., 2008). This pathway signals through Wnt3a, Dvl1, Gsk3β inhibition and APC, and is independent of transcription (Purro et al., 2008). Here, Wnt3a induces growth cone pausing with concomitant remodeling and growth cone expansion; these neuronal behaviors are regulated by microtubule organization. APC, in addition to being a component of the destruction complex (described above in the canonical-Wnt pathway), is a microtubule plus-end binding protein that captures the distal end of microtubules to the leading edge of the growth cone (Galjart, 2005). Wnt3a signals through Dvl1 and Gsk3β inhibition to induce the loss of APC from microtubule plus ends, which consequently abolishes microtubule directionality and arrests growth cone translocation (Purro et al., 2008). These data are consistent with previous findings that Wnt3 in the spinal cord regulates terminal arborization of innervating sensory neurons (Krylova et al., 2002), and presynaptic remodeling in mossy fibers in the mouse cerebellum (Hall et al., 2000). Although it has not yet been confirmed whether the same pathway is activated in these axons.

Together, these studies demonstrate that the canonical-Wnt pathway diverges at molecular intermediates with different functional outcomes. The identification of novel canonical-Wnt pathways further underscores the complexities of Wnt signaling and supports a call of a review on the nomenclature used to describe Wnt signaling (van Amerongen and Nusse, 2009).

Non-canonical-Wnt signaling

- *Wnt/Ca^{++} pathway*

The term Wnt/Ca^{++} pathway was first described in 2000 in response to a growing body of work that collectively supported an alternative Wnt model of signaling (Kuhl et al., 2000a). For example, Wnt5a switches from non-canonical to canonical-Wnt signaling when co-expressed with specific Fz receptors (He et al., 1997; Itoh et al., 1998) and also stimulates Ca^{++} transients, which is not a response associated with canonical signaling (Slusarski et al., 1997a). Wnt/Ca^{++}

signaling pathway can be activated by a subset of Wnt ligands that include Wnt-4, -5a, -5b and -11 (Kuhl et al., 2000b; Slusarski et al., 1997b; Slusarski et al., 1997a; Westfall et al., 2003). These specific Wnts interact with Fz2 (Slusarski et al., 1997b), and putatively Fzs-3, -4 and -6 (Kuhl et al., 2000b) to activate intracellular signaling through G-protein, Dvl, PLC, DAG and IP_3 to release intracellular Ca^{++} (Kuhl et al., 2000a; Semenov et al., 2007) (Figure 1.4). In turn, elevated Ca^{++} levels stimulate the Ca^{++} sensitive kinases PKC and CaMKII and the phosphatase CNA; PKC signaling through Cdc42 regulates actin dynamics and possibly cell-adhesion during cell separation and movement during gastrulation (Semenov et al., 2007). Calcium activation of CaMKII and CNA stimulates the transcription factor NFAT (nuclear factor of activated T-cells), which regulates *Xenopus* ventral patterning (Kohn and Moon, 2005; Saneyoshi et al., 2002). CaMKII activation also regulates specific kinases that inhibit β-catenin/TCF transcription, and therefore antagonizes canonical-Wnt signaling (Saneyoshi et al., 2002).

- *The planar cell polarity pathway*

The planar cell polarity (PCP) pathway also modulates actin cytoskeleton dynamics via Wnt/Fz/Dvl signaling, but the downstream molecular intermediates are different to those of the Wnt/Ca^{++} pathway (Figure 1.4), and the cellular outcomes include cell and tissue polarity, convergent extension movements, neural-tube closure and dendritogenesis (Ciani and Salinas, 2005; Salinas and Zou, 2008; Seifert and Mlodzik, 2007; Wang and Nathans, 2007). Phenotypic analyses and functional studies of *Drosophila* and vertebrate mutants reveal that a number of core components, including Fz and Dvl, interact to establish initial cellular polarity (Seifert and Mlodzik, 2007). Activation of the PCP pathway through Wnt/Fz binding leads to asymmetric enrichment of the core complex, which is critical for establishing polarity (Seifert and Mlodzik, 2007). The Wnt ligands known to activate PCP signaling are Wnt-5, -7a and -11 (Seifert and Mlodzik, 2007). Wnt-dependent Dvl recruitment to the membrane by Fz is regulated by kinases such as CK1, PKC and Par1, and within the cytoplasm multiple Dvl-dependent pathways are activated via the small GTPases Rac and RhoA, and the kinases ROCK and JNK, which together converge on the actin cytoskeleton to regulate cell polarity, cell motility and neurite outgrowth (Seifert and Mlodzik, 2007; Semenov et al., 2007; Wang and Nathans, 2007).

Although researchers have studied the effects of specific Wnt isoforms and their roles in activating specific pathways, it is not clear that pathway specificity is determined at the level of the ligand. Rather, as described above, the availability of receptors and co-receptors, intracellular signaling molecules and antagonists, which may be tissue specific or developmentally regulated are likely to play the pivotal role in specifying which Wnt pathway is activated (Gordon and Nusse, 2006; Kikuchi et al., 2007; Mikels and Nusse, 2006; van Amerongen and Nusse, 2009).

- *Frizzled Nuclear import pathway*

Receptor cleavage, endocytosis and transport to the nucleus are cellular strategies used to directly couple signaling events at the cell surface with transcription in the nucleus (Baron et al., 2002; Gomez-Ospina et al., 2006; Lin et al., 2001). During *Drosophila* NMJ development, Wg secreted from presynaptic terminals binds to DFz2 in the pre and postsynaptic membrane (Packard et al., 2002). In the postsynaptic muscle, DFz2 is subsequently endocytosed and cleaved at a conserved guatamyl endopeptidase cleavage site (Mathew et al., 2005) (Figure 1.4). Antibody labeling of the C- and N-terminals of DFz2 reveals punctate labeling of both domains at the NMJ, and in addition the C-terminus is found associated with chromosomal regions of proximal muscle nuclei, whereas the N-terminus locates peri-nuclear (Mathew et al., 2005). The nuclear transport of DFz2 is Wg-dependent, yet DFz2 endocytosis and cleavage events are Wg-independent events. Whilst Wg-dependent Fz nuclear import (FNI) and has only been observed postsynaptically, this signaling pathway regulates both pre- and postsynaptic development (Speese and Budnik, 2007). Further research is required to determine if the FNI pathway elicits a transcriptional response.

1.4.4.Regulation of Wnt signaling

The diverse functional outcomes mediated by Wnt signaling during development necessitate tight temporal and spatial regulation of the signaling pathways. The principle modes of Wnt antagonism are ligand sequestration in the ECM or binding with Fzs and/or co-receptors at the plasma membrane. Here I will briefly describe the most characterized Wnt antagonists with particular focus given to Dkk1 as I used it extensively to block Wnt signaling to explore the role of Wnt in synapse formation, disassembly and maintenance.

Soluble Frizzled-related proteins

Soluble Frizzled-related proteins (Sfrps) are structurally related to Fz receptors and have been principally characterized as robust Wnt antagonists (Bovolenta et al., 2008; Kawano and Kypta, 2003). Five Sfrp family members have been identified in the mouse genome (Sfrp-1 to -5), each containing conserved N-terminus CRDs that share 30-50% homology with Fz proteins (Melkonyan et al., 1997). The Sfrp C-terminus contains domains that appear to concede heparin sulphate proteoglycan (HSPG) binding, and formation and stabilization of Sfrp-Wnt complexes is enhanced by exogenous heparin (Uren et al., 2000). Direct binding of Sfrps to Wnt ligands via their CRDs blocks Wnt signaling by effectively sequestrating the ligand before receptor binding (Bafico et al., 1999; Bovolenta et al., 2008; Dennis et al., 1999; Uren et al., 2000; Wawrzak et al., 2007).

In addition to binding with extracellular Wnts, Sfrps interact with Fzs at the plasma membrane to form a nonfunctional complex, which directly blocks Wnt signal transduction at the receptor level

(Bafico et al., 1999). Sfrp3-Fz8 heterodimers have been captured by crystal structure determination demonstrating direct Sfrp-Fz interactions (Dann et al., 2001). Unfortunately, analyses of downstream molecular or cellular events were not within the scope of this paper so any functional outcomes of Sfrp3-Fz8 interactions remain unknown (Dann et al., 2001).

In contrast to Sfrp negative regulation of Wnt signaling, evidence supports a model for Sfrp potentiation of intracellular Wnt-signaling (Bovolenta et al., 2008). Sfrp1 binds with Fz1 in developing chick and *Xenopus* retinal ganglion cells (Rodriguez et al., 2005). In this cellular context, Sfrp1 positively regulates axon outgrowth without inhibiting Wnt signaling (Rodriguez et al., 2005). Furthermore, Sfrp2 potentiates Wnt mediated synaptogensis in immature rat hippocampal neurons (Davis et al., 2008). Potentiation of Wnt signaling in this context is lost as neurons mature, whereupon Sfrp2 inhibits exogenous Wnt (unpublished data from our lab). Additional complexity of Sfrp regulation of Wnt signaling was revealed in a study designed to test Sfrp-Wnt binding (Uren et al., 2000). Here the authors revealed that Sfrp1 exhibits a biphasic effect on *Drosophila* Wg signaling, whereby low Sfrp1 concentrations potentiate canonical-Wnt signaling as demonstrated by increased cytosolic Armadillo (the β-catenin homologue) (Uren et al., 2000). It has been suggested that Sfrp1 contains low-affinity and high-affinity Wg binding sites, which inhibit or potentiate Wg signaling respectively (Kawano and Kypta, 2003). However, full characterization of these putative sites has yet to be determined and the mechanisms behind Sfrp dual regulation of Wnt signaling remains unresolved. Different Sfrp isoforms may also regulate one another; during kidney formation, Sfrp1 inhibits Wnt4 mediated tubule development yet Sfrp2 rescues the defect (Yoshino et al., 2001). Whilst Sfrp function appears contradictory under different cellular contexts, Sfrps are widely accepted as general Wnt antagonists.

Dickkopf proteins

Dickkopf proteins (Dkk) are a family of secreted glycoproteins that include Dkk-1, -2, -3 and -4 in vertebrates (Krupnik et al., 1999). Dkk-1, -2 and -4 contain two conserved CRDs, which mediate canonical-Wnt signaling via direct binding with LRP5/6 (Bafico et al., 2001; Glinka et al., 1998; Mao et al., 2001; Semenov et al., 2001). The CRDs could potentially provide a site for Dkk-Wnt binding, but this has not yet been fully characterized. Dkk3 is a divergent member of the Dkk family (Glinka et al., 1998) that is not involved in the regulation or function of Wnt signaling (Niehrs, 2006).

In addition to LRP5/6, Dkks bind to Kremen proteins (Mao et al., 2002). Kremen proteins-1 and -2 are high affinity Dkk receptors that synergize Dkk1 inhibition of Wnt signaling (Mao et al., 2002). Kremens are transmembrane proteins that bind directly with Dkk and are required for Dkk1 mediated *Xenopus* head induction (Davidson et al., 2002). Kremen forms a ternary complex with Dkks and LRP5/6, which induces rapid endocytosis of the complex (within 5 minutes of Dkk1 application) and the consequent removal of LRP5/6 from the plasma

membrane (Figure 1.5) (Mao et al., 2002). Western blot analyses reveal a loss of total LRP5/6 following Dkk1 application suggesting that internalized LRP5/6 is degraded (Mao et al., 2002).

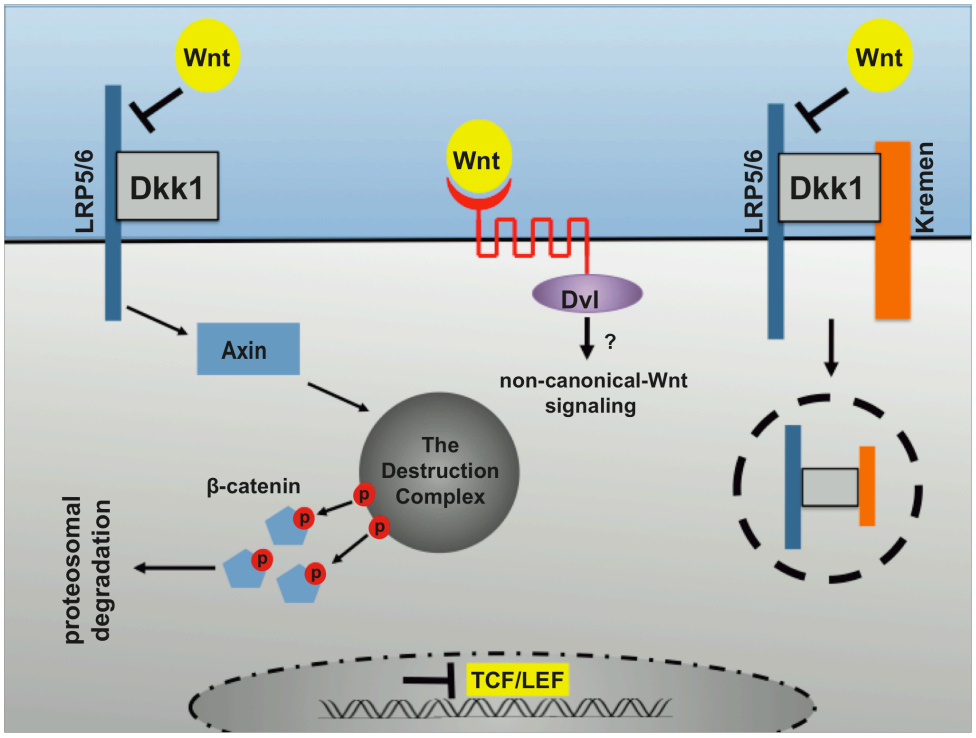


Figure 1.5. Dkk1 blocks canonical-Wnt signalling. Dkk1 binds to LRP5/6 with high affinity at a domain that is distinct from Wnt/LRP binding. Dkk1/LRP binding prevents Wnt/LRP binding, possibly by inducing LRP5/6 conformational changes such to prevent Wnt interactions. Dkk1 blocks formation of the Wnt induced Fz/LRP complex and thus blocks canonical-Wnt signalling at the receptor level. In addition, Dkk1 binding with LRP5/6 induces intracellular disassociation between LRP5/6 and Axin. This potentially makes Axin available to the destruction complex. Dkk1 also binds to Kremen proteins with high affinity to form a ternary complex that includes LRP5/6. This complex is rapidly endocytosed (within 5 minutes of Dkk1 application) and LRP5/6 is degraded. Frizzled receptors remain intact at the plasma membrane in the presence of Dkk1 and potentially available for non-canonical-Wnt signalling.

Dkk-1 and -4 antagonize the canonical-Wnt pathway, whereas Dkk2 can agonize or antagonize canonical-Wnt signaling depending on the cellular context (Fedi et al., 1999; Krupnik et al., 1999; Niehrs, 2006; Wu et al., 2000). Dkk1 binds to LRP5/6 with significantly greater affinity compared to Dkk2 or 4 (Niehrs, 2006). Dkk1 and Dkk2 bind to LRP5/6 via C-terminal domains that activate canonical-Wnt signaling (Brott and Sokol, 2002). Critically, Dkk1 contains an N-terminal domain that suppresses LRP5/6 activation (Brott and Sokol, 2002). In contrast, Dkk2 does not contain this N-terminal domain and acts as a weak activator of LRP5/6 dependant Wnt signaling (Brott and Sokol, 2002). However, LRP5/6 needs to be strongly expressed for this to occur and the Kremen receptor must be absent (Brott and Sokol, 2002; MacDonald et al., 2009; Mao and Niehrs, 2003). Dkk2 antagonizes canonical-Wnt signaling in the presence of Kremen, which as described below, induces endocytosis of LRP5/6 (Mao and Niehrs, 2003; Mao et al., 2002).Dkk1, the most characterized protein of the Dkk family, is a potent inhibitor of canonical-Wnt signaling (Figure 1.5). The first studies to examine Dkk1 function were performed in

Xenopus embryos. In-situ hybridization analyses revealed that Dkk1 is expressed in the Spemann's organizer during early and late gastrula when head induction occurs (Glinka et al., 1998). Overexpression of Dkk1 induced significant expansion of anterior head structures, and injection of anti-Dkk1 antibodies caused microcephaly (Glinka et al., 1998). Indeed, this phenotype gave its namesake to the protein; dickkopf translates to "big head" in German. Dkk1 binds to a distinct extracellular domain of LRP5/6 (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001) that is not required for Wnt or Fz binding (Mao et al., 2001), and does so with greater affinity, and at lower concentrations, compared to interactions between Wnts and Fzs (Zorn, 2001). Co-immunoprecipitation experiments demonstrate physical interactions between Dkk1 and LRP5/6 (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001) and further protein binding assays reveal Dkk1 blocks the formation of Wnt-induced Fz-LRP5/6 complexes (Semenov et al., 2001). The above studies suggest that Dkk1 does not compete for the same LRP5/6 binding site as Wnt ligands. It is proposed that Dkk1-LRP5/6 binding alters LRP5/6 conformation such to prevent Wnt interactions, and subsequent activation of canonical-Wnt signaling (Mao et al., 2001; Zorn, 2001). Furthermore, Dkk1 binding with LRP5/6 may also induce intracellular disassociation between LRP5/6 and Axin, therefore releasing Axin into the cytoplasm making it available to the destruction complex and subsequent β-catenin degradation (Figure 1.5) (Zorn, 2001).

Whether Dkk1 binds with Kremen and LRP5/6 to induce LRP5/6 internalization, or binds solely to LRP5/6 to block Wnt binding, Fz receptors remain intact and are potentially available for Wnt interactions (Figure 1.5). Therefore, Dkk1 could provide a mechanism for specifying non-canonical Wnt signaling (Zorn, 2001). Indeed, Dkk1 stimulates JNK in cardiogenic explant tissue (Pandur et al., 2002) and enhances Wnt3a-mediated neurite outgrowth in Ewing tumor cells via Fz3-JNK signaling (Endo et al., 2008). A role for Dkk1 balancing canonical *versus* non-canonical-Wnt signaling has yet to be demonstrated in-*vivo* and whether such a role exists in CNS development and function is unknown.

Wnt inhibitory factor 1

Wnt inhibitory factor 1 (WIF1) was original identified as an expressed sequence tag from the human retina, and the deduced amino acid sequence revealed a conserved N-terminal signal sequence; the WIF domain (Hsieh et al., 1999), which is also found in the extracellular domain of the RYK family of tyrosine kinase receptors (described above) (Patthy, 2000). WIF1 is a secreted factor that is dynamically expressed in the *Xenopus* embryo and contributes to correct somitogenesis, and anterior brain structure formation in both *Xenopus* and Zebra fish (Hsieh et al., 1999). WIF1 overexpression in *Xenopus* embryos phenocopies XWnt8 blockade (Hoppler et al., 1996; Hsieh et al., 1999). Furthermore, WIF1 blocks XWnt8 activity in a dose dependent manner (Hsieh et al., 1999). In-vitro analyses demonstrate that WIF1 binds directly and reversibly to XWnt8 with high binding affinity and competes with the Fz2-CRD for XWnt8 binding (Hsieh et al., 1999). More recently, a role for WIF1 has been identified in skeletal

development and cardiogenesis (Buermans et al.; Surmann-Schmitt et al., 2009) and reduced tumor growth in cancer cells (Kawakami et al., 2009; Yee et al., 2010).

Cerberus

Cerberus is another secreted Wnt antagonist that belongs to the same class of Wnt inhibitors as Sfrps and WIF1 (Kawano and Kypta, 2003); like Sfrps and WIF1, Cerberus sequesters extracellular Wnt and prevents Wnt/receptor binding at the membrane (Piccolo et al., 1999). Alongside Dkk1, Cerberus is expressed in the Spemann's organizer and accordingly, overexpression induces ectopic head formation (Bouwmeester et al., 1996; Glinka et al., 1998). Cerberus also blocks BMP and Nodal signaling by binding with each ligand at independent sites (Piccolo et al., 1999).

1.4.5.Wnt signaling in the CNS

Wnts regulate a number of critical cellular processes during embryonic CNS development including cell proliferation and cell fate, neuronal polarity and migration, neurite outgrowth, axon guidance and branching, and synapse formation (Budnik and Salinas, 2011; Ciani and Salinas, 2005; Ille and Sommer, 2005; Salinas and Zou, 2008). Importantly, a number of Wnts and regulators of Wnt signaling continue to be expressed in the adult CNS (Coyle-Rink et al., 2002; Gogolla et al., 2009; Shimogori et al., 2004), which raises the interesting question of whether Wnt signaling is involved in ongoing synaptic function in the mature CNS. In this final introductory section, I will briefly describe the role of Wnt signaling during early neural circuit assembly, before discussing in greater detail the role of Wnt signaling in the formation and function of synapses.

Wnt signaling in CNS patterning and neural connectivity

The genesis of neuronal connections and establishment of function neural circuits fundamentally requires the correct cell type in the correct location. The anterior to posterior (A-P) axis of the early CNS is established via a Wnt gradient which acts as a positional cue to instruct progenitor cells and determine cell fate (Kiecker and Niehrs, 2001; Nordstrom et al., 2002). Wnt is released from posterior cells of the neural tube and the antagonist Dkk1 is secreted from the anterior neural tube, to refine the gradient, which regulates neural pattering (Glinka et al., 1998; Hashimoto et al., 2000; Kiecker and Niehrs, 2001; Mukhopadhyay et al., 2001). Ectopic Wnt induces expression of posterior neural structures in *Xenopus* (McGrew et al., 1997; McGrew et al., 1995) and conversely, ectopic expression of Dkk1 induces an expansion of anterior brain structures (Mukhopadhyay et al., 2001). A Wnt gradient also regulates dorsal-ventral patterning of the early CNS from the forebrain to the spinal cord (Gunhaga et al., 2003) and continued local expression during embryonic CNS development regulates early hippocampal development (Galceran et al., 2000; Lee et al., 2000). The

canonical-Wnt pathway is implicated in all of the above examples and most probably regulates neural tube pattering by regulating cell fate.

Axon guidance is tightly regulated by a combination of attractive and repulsive cues, which together steer the axon along the correct trajectory by regulating cytoskeletal dynamics (Huber et al., 2003). Wnt signaling acts in conjunction with a host of axon guidance molecules such as semaphorins, netrins and ephrins to ensure axons project to their correct target cells (Bovolenta et al., 2006; Dickson, 2002). Specifically, Wnt/Ryk (or Derailed, the *Drosophila* homologue to Ryk) signaling directs commissural axons across the midline in *Drosophila* (Yoshikawa et al., 2003) and longitudinal axon outgrowth of corticospinal neurons in mouse (Liu et al., 2005b). Commissural axons navigating the mouse corpus callosum require Wnt/Ryk signaling (Keeble et al., 2006), as does topographic mapping along the lateral-medial axis in the chick optic tectum (Schmitt et al., 2006). Evidence from mouse and *Drosophila* demonstrate that Wnt regulates the repulsion of axons by signaling via Ryk (Liu et al., 2005b; Yoshikawa et al., 2003), whilst Wnt interactions with Fz act as an attractant cue (Lyuksyutova et al., 2003). The evidence therefore supports a model whereby the expression of receptor type at the growth cone determines whether Wnt signaling acts as a repulsive or attractant cue to direct axon guidance. In both cases, downstream molecular intermediates converge on the cytoskeleton to induce changes in axon branching, stability, elongation and diameter, and growth cone turning (Bovolenta et al., 2006; Ciani and Salinas, 2005; Krylova et al., 2002; Purro et al., 2008).

In addition to axonal behaviors, Wnts regulate dendrite outgrowth and arborization (Rosso et al., 2005; Wayman et al., 2006; Yu and Malenka, 2003). Activity dependent Ca^{++} signaling within dendrites stimulates transcription of Wnt2 via CREB activation, which in turn stimulates dendritic outgrowth (Wayman et al., 2006). Importantly, this study demonstrated that the secreted Wnt inhibitor Wif blocked activity-dependent dendritic growth. Another member of the Wnt family, Wnt7b, has been shown to regulate dendritic development and morphology (Rosso et al., 2005). In mouse hippocampal dendrites, Wnt7b signals via Dvl1, Rac and JNK in the PCP pathway to affect cytoskeletal changes, which mediate dendrite length and branching (Ciani and Salinas, 2005; Rosso et al., 2005). In a separate study, a role for activity-dependent Wnt release and β-catenin dependent dendritic development was suggested (Yu and Malenka, 2003). Here, the dendritic effects were mediated by β-catenin stability, but were independent of transcription (Yu and Malenka, 2003). Collectively, these works reveal a role for activity dependent Wnt transcription and release, and a mechanism for regulating dendritic development, however, whether Wnt7b is also secreted in an activity-dependent manner has yet to be realized, and the role of β-catenin in dendritogenesis has yet to be elucidated.

Wnt signaling therefore plays multiple roles in early CNS pattering and the above description clearly demonstrates how different pathways are temporally and spatially activated to achieve diverse outcomes. What is also evident is that neurites have been exposed to Wnts prior to synaptogenesis and may therefore already be receptive, or “primed’ to Wnt signaling.

Wnt signaling in the formation and function of synapses

It was previously posited that Wnt signaling primes neurites to become competent for synaptogenesis without directly regulating the assembly of synapses (Waites et al., 2005). However, the use of mutant *Drosophila* and mouse models together with extensive in-vitro approaches have provided compelling evidence that Wnt signaling directly regulates, and is a requirement for, correct synapse assembly at the NMJ and mossy-fiber/granule cell synapses in the cerebellum. Furthermore, studies of the hippocampus demonstrate a role for Wnt signaling in the formation and function of immature and mature synapses. Here I will discuss the role of Wnt signaling in these three regions and what is known about the underlying molecular mechanisms.

- *The neuromuscular junction*

The formation and function of synapses requires a coordinated molecular dialogue between the pre- and postsynaptic compartments. Wnt signaling acts as a bi-directional signal across the *Drosophila* NMJ and is required for the correct assembly of both pre- and postsynaptic structures and ongoing synaptic plasticity (Ataman et al., 2008; Korkut et al., 2009; Packard et al., 2002; Wu et al., 2010). Wnt1/Wingless (Wg) is secreted from fly motoneurons in an activity dependent manner and conditional Wg mutants, who fail to release Wg presynaptically during development, display significant reductions in bouton number (Packard et al., 2002). Furthermore, the boutons that do form display deficits in active zone structure, morphology and contain abnormal cytoskeletal organization such as splayed microtubule bundles (Ataman et al., 2008; Miech et al., 2008; Packard et al., 2002). Postsynaptically, the PSD is misshapen and glutamate receptors are mislocalized (Packard et al., 2002). In addition, Wg mutants form “ghost boutons”, which are presynaptic structures without active zones or opposing postsynaptic specializations (Ataman et al., 2008; Packard et al., 2002). Therefore, disruption of presynaptic Wg release induces profound synaptic deficits on both sides of the synapse suggesting anterograde signaling, and possibly autocrine mechanisms.

Disruption of postsynaptic Wg signaling by downregulation of the DFz2 interacting PDZ domain dGRIP, or mutations in DFz2 or other components of the signaling pathway, also induces severe pre- and postsynaptic deficits that are phenotypically similar to presynaptic Wg mutants; bouton numbers are significantly reduced and the formation of “ghost boutons” occurs (Ataman et al., 2008; Mathew et al., 2005; Mosca and Schwarz, 2010). Where postsynaptic specializations do form, Discs-Large (which is related to PSD95) is aberrantly localized, as are Glu-Rs (Mosca and Schwarz, 2010; Packard et al., 2002). These data demonstrate that postsynaptic Wg signaling is required for presynaptic development.

In the presynaptic bouton, Arrow and Shaggy, which are the *Drosophila* homologues for LRP5/6 and Gsk3β respectively, mediate Wg signaling (Packard et al., 2002). Downstream of Shaggy the synaptogenic effects are independent of transcription suggesting activation of a divergent

canonical Wnt pathway (Miech et al., 2008). Accordingly, loss of presynaptic Wg signaling alters the distribution of the MAP1B homologue Fusch on microtubules (Packard et al., 2002), which is consistent with a role for Wnt regulation of microtubule organization by a divergent-canonical pathway in vertebrates (Ciani et al., 2004; Purro et al., 2008). On the postsynaptic side Wg binds to DFz2 to activate the nuclear import pathway (Figure 1.4), which is required for assembling the postsynaptic apparatus (Ataman et al., 2008; Mathew et al., 2005; Mosca and Schwarz, 2010).

Wg signaling at the *Drosophila* NMJ is also involved in activity-dependent synaptic plasticity (Ataman et al., 2008). Patterned stimulation of the motoneuron induces transcription/translation dependent outgrowth of motile presynaptic filopodia, the *de novo* formation of boutons and differentiation/maturation of existing presynaptic varicosities (Ataman et al., 2008). Furthermore, frequency potentiation of spontaneous neurotransmitter release is enhanced (Ataman et al., 2008). Evoked activity stimulates Wg release from boutons and subsequent activation of the pre and postsynaptic Wnt pathways described above. Critically, Wg mutations block activity-dependent presynaptic bouton formation and Wg overexpression accelerates the rate of activity dependent synaptic growth (Ataman et al., 2008). In addition to Wg and DFz2, Wnt5 and Derailed (Drl) are active at the NMJ (Liebl et al., 2008). Wnt5 mutants exhibit reductions in the frequency of spontaneous excitatory junctional currents (EJCs) and the amplitude of evoked EJCs, suggesting both pre and postsynaptic functional deficits (Liebl et al., 2008). The extensive characterization of Wnt signaling at the *Drosophila* NMJ demonstrates that Wg stimulates different pathways in the pre- and postsynaptic terminals and bidirectional activation of the pathways by the same Wnt ligands provide a molecular dialogue to ensure synchronized pre and postsynaptic assembly and function.

In contrast to the above data, Wnt signaling is reported as an anti-synaptogenic factor at the *C. Elegans* NMJ (Klassen and Shen, 2007). In this case, the Wnt proteins Lin-44 and Egl-20 spatially regulate the assembly of synapses and the stereotyped patterning of neural connections along the DA9 motoneuron by inhibiting synaptogenesis in proximal regions. The anti-synaptogenic effects of Wnt reported here are mediated by signaling through Lin17/Fz and Dvl1 (Klassen and Shen, 2007). Lin-44 mutants exhibit ectopic synapses in the proximal regions of the DA9 motorneuron and conversely, overexpression on Lin-44 in cells neighboring the distal region inhibits synapse assembly (Klassen and Shen, 2007). Importantly, the data from this study suggests that presynaptic release of Wnt/Lin-44 regulates the localization of Lin17/Fz. This anti-synaptogenic role for Wnt corroborates a study that suggests exogenous Wnt5a can block the formation of excitatory synapses in cultured hippocampal neurons derived from embryonic rats (Davis et al., 2008). These studies demonstrate that under certain developmental conditions, Wnt signaling negatively regulates neuronal behaviors, as described above during axon guidance.

In vertebrates, Wnt signaling cooperates with the HSPG Agrin to promote postsynaptic AchR clustering. In the developing chick limb bud, Wnt3 is expressed by motoneurons innervating newly formed muscle fibers (Henriquez et al., 2008). Wnt3 induces the formation of AchR

microclusters *in-vivo* and *in-vitro* and blockade of Wnt signaling by transplantation of cells expressing Sfrp1 into the developing limb bud reduces AchR clustering (Henriquez et al., 2008). Agrin subsequently converts microclusters into the larger AchR plaques that are characteristic of developing NMJs (Henriquez et al., 2008). Wnt3 signals via Rac1 and Dvl1 to mediate AchR clustering (Henriquez et al., 2008). In zebrafish, Wnt11r binds with and activates MuSK, which also signals through Rac (Jing et al., 2009). In accordance with antagonistic Wnt activities previously described, canonical-Wnt3a signaling has been shown to reduce AchR clustering at the mouse NMJ by inhibiting the expression of *Rapsyn*, which is an AchR binding protein that stabilizes the clusters (Wang et al., 2008).

Wnt signaling is therefore a conserved mechanism in NMJ development, and further research will determine whether the different molecular intermediates represent tissue or species specific mechanisms, or whether different Wnts are co-expressed to regulate different aspects of synapse development.

- *The cerebellum*

Differentiating granule cells in the mouse cerebellum *in-vivo* express Wnt7a during the peak time of synaptogenesis (Lucas and Salinas, 1997). Indeed it was this study that first revealed a link between Wnt signaling through Gsk3β and increased clustering of Synapsin1, which is a hallmark of presynaptic differentiation. Furthermore, the authors posited a model for Wnt mediated synaptogenesis between granule cells and mossy fibers through cytoskeletal reorganization (Lucas and Salinas, 1997). Individual mossy fibers synapse with multiple granule cells to form morphologically distinct structures referred to as glomerular rosettes. This name reflects the complex multisynaptic structure that forms once the mossy fiber growth cone reaches the dendrites of granule cells and interdigitates between them (Hamori and Somogyi, 1983). Endogenous Wnt7a is released from granule cell and acts as a retrograde signal to induce extensive axonal remodeling and Synapsin1 clustering at synaptic sites within the glomerular rosette (Hall et al., 2000). Wnt7a mutant mice form significantly less complex glomerular rosettes and exogenous Sfrp blocks axonal remodeling (Hall et al., 2000). Furthermore, inhibition of Gsk3β by lithium mimics the effects exogenous Wnt7a on neuronal remodeling by mediating microtubule dynamics and also the effects of Synapsin1 clustering (Hall et al., 2002; Hall et al., 2000).

Overexpression of Dvl1 also mimics Wnt7a effects by increasing the clustering of synaptic proteins and conversely, Dvl1 mutant mice exhibit significantly fewer synaptic clusters (Ahmad-Annuar et al., 2006). Moreover, Wnt7a/Dvl1 double mutant mice exhibit a more severe phenotype compared to single mutants (Ahmad-Annuar et al., 2006). In addition to stimulating the accumulation of synaptic proteins, Wnt7a/b through Dvl1 regulates synaptic vesicle recycling in the cerebellum (Ahmad-Annuar et al., 2006). Addition of exogenous Wnt7b increases the number of recycling sites and the size of the recycling pool, whereas Dvl1 mutant mice exhibit significantly fewer and smaller synaptic vesicle recycling sites (Ahmad-Annuar et

al., 2006). The deficit in presynaptic neurotransmitter release sites is reflected in electrophysiological recordings as a significant reduction in the frequency of mEPSCs (Ahmad-Annuar et al., 2006). Therefore, together these studies demonstrate that Wnt7a/b signals in the cerebellum via Dvl1 and Gsk3β to stimulate presynaptic assembly of functional nascent sites. Whilst this work suggests activation of the canonical-Wnt signaling, further characterization of the pathway remains poorly understood.

- *The hippocampus*

A number of Wnts, including Wnt-3a, -5a, -7a and -7b and cognate receptors are expressed in the hippocampus during the peak of synaptogenesis and in the adult brain (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Davis et al., 2008; Farias et al., 2009; Gogolla et al., 2009; Rosso et al., 2005; Sahores et al., 2010; Shimogori et al., 2004; Varela-Nallar et al., 2009). Exogenous Wnt7b and ectopic Dvl1 expression both increase the clustering of presynaptic markers in cultured hippocampal neurons (Ahmad-Annuar et al., 2006), an effect that is mimicked by the Gsk3 inhibitor lithium (Davis et al., 2008). Sfrps and Dkk1 both block Wnt7a/b mediated presynaptic differentiation (Ahmad-Annuar et al., 2006; Davis et al., 2008) and accordingly, Wnt7a-Dvl1 mutant mice exhibit significantly fewer synaptic sites (Ahmad-Annuar et al., 2006). In accordance with the cerebellar studies above, these data suggest activation of the canonical-Wnt pathway regulates the assembly of presynaptic sites. A recent study has demonstrated that Wnt7a binds to the CRD of Fz5 to stimulate synapse assembly (Sahores et al., 2010). Importantly, endogenous Wnt signaling mobilizes Fz5 to the cell surface at synaptic sites in an activity-dependent manner (Sahores et al., 2010). Moreover, blockade of Fz5 activity inhibits the increase in synapse number induced by high frequency stimulation, demonstrating a role for endogenous Wnt signaling during activity-dependent synaptogenesis (Sahores et al., 2010).

Postsynaptically, research suggests that Wnt5a stimulates PSD95 clustering via the PCP pathway, and $GABA_A$-R through CaMKII activation (Cuitino et al., 2010; Farias et al., 2009). In addition, the same group has postulated a mechanism for Wnt5a-mediated spinogenesis via Ca^{++} signaling (Varela-Nallar et al., 2010). However, Wnt5a has also been shown to inhibit the clustering of synaptic markers (Davis et al., 2008). Therefore, the role of Wnt5a is controversial in hippocampal synapse assembly. As previously discussed, Wnt signaling is highly complex and is determined and regulated by an array of factors including receptor type; the postsynaptic receptor for Wnt5a signaling in the hippocampus has yet to be elucidated. Further work is required to determine whether Wnt5a acts positively or negatively in regulating hippocampal synaptogenesis and whether it stimulates postsynaptic differentiation of both excitatory and inhibitory synapses.

During the writing of this thesis manuscript, research from our lab was published that demonstrates Wnt7a positively regulates the number of functional excitatory synapses via postsynaptic signaling (Ciani et al., 2011). Importantly, Wnt7a does not affect the number or function of inhibitory synapses. These findings reveal Wnt7a as a mediator of balancing

excitatory *versus* inhibitory synaptic transmission in the rat hippocampus. Loss and gain of function analyses show that Wnt7a signals directly to dendritic spines to stimulate spine growth and synaptic strength via Dvl1 and CaMKII (Ciani et al., 2011). Interestingly, postsynaptic activation of Wnt signaling by overexpression of Dvl1 does not increase the number of spines; only their morphology, and the frequency and amplitude of miniature EPSCs are affected (Ciani et al., 2011). In contrast, bath application of Wnt7a significantly increases the number of excitatory pre- and postsynaptic sites (Ciani et al., 2011). As previously described, Wnt7a stimulates presynaptic differentiation via Dvl/Gsk3β signaling as well as regulating release probability. Taken together with our recent publication, these data suggest that Wnt7a signals presynaptically via Dvl1/Gsk3β to stimulate synapse assembly, which subsequently stimulates postsynaptic differentiation. Wnt7a then signals directly to the nascent postsynaptic site via Dvl1/CaMKII to stimulate spine growth and synaptic transmission. This model suggests Wnt7a signals bidirectionally to stimulate excitatory synaptogenesis, maturation and function. Such a role is consistent with the *Drosophila* model (Ataman et al., 2008; Packard et al., 2002), albeit via activation of different Wnt pathways, and purports a conserved role across the synapse for Wnt signaling for synapse assembly and function.

Compelling evidence is now amassing to support a model for Wnt signaling in synaptic plasticity in the mammalian hippocampus. Studies demonstrate that Wnt expression is stimulated by neuronal activity (Ataman et al., 2008; Wayman et al., 2006; Yu and Malenka, 2003). Moreover, mice exposed to environmental enrichment (EE) show enhanced levels of postsynaptic Wnt7a/b expression in the hippocampus, which is associated with significant increases in synapse number and extensive synaptic remodeling of the mossy fiber-CA3 synapse (Gogolla et al., 2009). The authors also demonstrate by immunofluorescence that excitatory activity increases Wnt7a/b expression levels *in-vivo*. The effects of EE are mimicked by localized application of Wnt7a and are blocked by local injection of Sfrp (Gogolla et al., 2009). Importantly, *in-vivo* delivery of Sfrp reduced the number of synaptic sites compared to control mice further supporting the existence of endogenous Wnt activity in the hippocampus. These data are in accordance with the cerebellar studies that reveal Wnt7a as a retrograde synaptogenic factor that regulates presynaptic remodeling and synapse assembly (Hall et al., 2000). Critically, this study demonstrates that Wnt7a/b is expressed *in-vivo* in response to enriched behavioral paradigms, and that Wnt signaling structurally modifies neuronal circuits in an activity-dependent manner.

Functional analyses further support a role for Wnt signaling in synapse plasticity and regulation of excitatory neurotransmission. NMDA-R activation through tetanic stimulation induces release of Wnt3a in hippocampal slices (Chen et al., 2006). Furthermore slice incubation with Wnt3a potentiates LTP and Fz8 blockade reduces LTP (Chen et al., 2006). Intracellular activation of canonical-Wnt signaling by a synthetic small molecule agonist or bath application of Wnt-3a or -7a increases basal and evoked activity and the frequency of spontaneous and miniature EPSCs, which is indicative of enhanced neurotransmitter release (Avila et al., 2010; Beaumont et al., 2007; Cerpa et al., 2008). These data support findings from studies of the cerebellum that

reveal a decrease in neurotransmission in double mutant mice for Wnt7a and Dvl1 (Ahmad-Annuar et al., 2006) and are consistent with a role for Dvl binding with Synaptotagmin to regulate SV exo- and endocytosis (Kishida et al., 2007). Increased neurotransmission by Wnt3a or intracellular Wnt agonist activity appears to follow a divergent canonical pathway that mobilizes Ca^{++} and is independent of transcription (Avila et al., 2010; Beaumont et al., 2007). Further research will elucidate the precise molecular mechanisms stimulated by Wnt signaling during activity paradigms and additional *in-vivo* analyses will determine the role of Wnt-mediated synaptic plasticity in the developing and adult brain.

1.5. Thesis Aims

Overwhealming evidence supports a role for Wnt-mediated synaptogenesis and synapse function. Wnt signaling is immensely complex and the full molecular details of how Wnt ligands stimulate the assembly of synapses in the mammalian hippocampus remain elusive. We know that Wnt signals through Dvl1 and Gsk3β to induce the clustering of key synaptic markers, which suggests activation of the canonical-Wnt pathway, but the downstream events past Gsk3β have yet to be characterized. This presented the opportunity to investigate how blockade of canonical-Wnt signaling affects synapse assembly. As is often the case in scientific research, serendipity played an important role in shaping this study. Blockade of endogenous canonical-Wnt signaling induced a rapid loss of synaptic sites, which paved the way forward to examine the role of Wnt as a modulator of synaptic maintenance.

The aims of this thesis were:

a. To examine whether Wnt7a is a pan-synaptogenic factor or if it preferentially stimulates the assembly of excitatory synapses

b. To characterize the signaling pathway activated during Wnt-mediated synapse assembly

c. To investigate the effects of acute Wnt blockade in young and mature neuronal cultures

d. To plot the response of a population of synapses to Wnt blockade and understand how quickly synapses disassemble in the absence of endogenous Wnt

CHAPTER 2:

2. Materials and methods

2.1. Dissociated hippocampal neuron cultures

Dissociated hippocampal cultures were prepared according to the Banker protocol (Banker and Cowan, 1977; Kaech and Banker, 2006). See section 2.11 for details of solutions for isolation and culture of neurons. Animals were housed and sacrificed in accordance with Home Office regulation. Brain tissue was obtained from E18 Sprague-Dawley rat embryos. Embryos were removed and their hippocampi quickly and carefully dissected out of the brain using sterilized tools. Hippocampi were kept in ice-cold Hank's balanced salt solution (HBSS) (Gibco) until the required number was obtained. Hippocampi tissue was dissociated chemically and mechanically; first by 18 minutes incubation in 0.5% trypsin (Invitrogen) at 37°C, then by tituration in 3 mL of cold HBSS using flame polished Pasteur pipettes. The tissue was carefully washed 3 times in the same solution between trypsin treatment tituration steps. Cell density was calculated using a haemocytometer. Cells were plated to the required density onto sterile 13mm or 24mm acid-cleaned glass coverslips coated with poly-L-lysine (Sigma) (1µg/mL in borate buffer) over night and then carefully washed in water. 24 mm coverslips were used for time-lapse experiments and 13 mm coverslips were used for all other experiments. Ten 13 mm coverslips, or three 24mm coverslips were placed into 60 mm plastic dishes (P60) (Corning), and incubated at 37°C in 3 mL of plating solution prior to cell plating. Plated cells were incubated for 2-hours in plating solution; the solution was then aspirated and replaced with warm culture solution. Cultured cells were incubated in 95% O_2 with 5% CO_2 at 37°C until required. Every 3-days, approximately one third of the culture solution was replaced with fresh culture solution.

- *Plating densities*

For the majority of immunofluorescence experiments, cells were plated at low-density (10 cell/mm^2). For electronmicroscopy analysis experiments, cells were plated at medium-density (30 cell/mm^2). For AMAXA and Lipofection2000 transfection experiments, cells were plated at high-density (70 cell/mm^2). For Western blot, 800,000 neurons (70 cell/mm^2) were plated directly onto the base of P60 plastic dishes (Corning), which had been coated with poly-L-lysine (1µg/mL in borate buffer).

2.2. Production of Wnt7b conditioned media

Wnt7b conditioned media was prepared from Rat1B cells stably transfected with a pLNCX retroviral vector containing HA-tagged Wnt7b cDNA. Cells were incubated in P60 plastic dishes

(Corning) with 3 mL of growth media at 37°C in 95% O_2 with 5% CO_2 until 70-80% confluent. Growth media was then exchanged with serum free media, and cells were cultured for a further 16-18 hours to condition the media. Freshly conditioned media was added to neuronal cultures immediately without storing. The level of Wnt7b protein in the conditioned media was determined by Western blot analyses. When recombinant Wnt7a became commercially available, I started to use this protein for all experiments as Wnt7a and Wnt7b stimulate synaptic differentiation at comparable levels (Ahmad-Annuar et al., 2006).

2.3. Western blot

In addition to determine the level of Wnt7b protein in conditioned media, Western blots were used to determine the levels of synaptic proteins in hippocampal cell lysates after treatment with Wnt7a/b, Dkk1 or control media.

Cells were cultured in P60 dishes until required then washed quickly in ice-cold phosphate buffer solution (PBS). Cells from each dish were lysed in 200 µL of ice-cold RIPA buffer containing phosphatase inhibitors (Sigma) then scraped from the dish and transferred to an eppendorf tube. Cells were then triturated using a 25G syringe and left on ice for 10-minutes. The samples were centrifuged at maximum speed using a benchtop centrifuge for 2-minutes and the supernatant retained. 50 µL of loading buffer was added to each 200 µL of supernatant and the samples were boiled in a heat-block at 100°C for 3-minutes.

Prior to running a SDS gel, total protein levels were determined by the Lowry assay to ensure equal amounts of protein were loaded. 10 µg of protein were run on SDS-PAGE (10% or 15% acrylamide depending the molecular weight of the protein being assayed) for ~20-minutes at 80V for the samples to run through the stacking gel and reach the resolving gel, then ~45-minutes at 125 V. The gel was then transferred to a nitrocellulose membrane (Hybond C, Amersham) and run overnight at 40 mA. The membrane was washed twice in dH_2O then blocked with 5% milk powder in TBST for 1-hour at room temperature with gentle shaking. Primary antibodies, diluted in 5% milk powder in TBST, were applied for 1-hour at room temperature with gentle shaking. Membranes were gently washed three times in dH_2O before exposure to secondary antibodies for 1-hour at room temperature with gentle shaking. After further washing, protein levels were detected using chemiluminescence (ECL) solution (Amersham) according to manufacturers instructions.

The ratio of a given synaptic protein to loading control (Erk) was determined by measuring the intensity of each band using ImageJ (http://rsbweb.nih.gov/ij). A colleague in the lab (Silvia Purro) performed this analysis for our joint publication (submitted at the time of writing).

2.4. Transfection methods

- *Lipotransfection*

Lipfectamine2000® (Invitrogen) was used to transfect hippocampal neurons with a DNA construct that expresses eGFP (enhanced green fluorescent protein) under the control of the CMV promoter in order to test the efficacy of ActinomycinD in blocking transcription. Hippocampal neurons were transfected at 5-7 DIV with either eGFP-expressing (Invitrogen) or empty vector. For each P60 dish to be transfected, 2 µL of Lipofectamine2000 reagent was added to 200 µL of Opti-MEM (Gibco) and incubated for 5 minutes at room temperature. This solution was then added in a drop-wise manner to a 200 µL of Opti-MEM containing 4 µg of eGFP DNA or control DNA. The mixture was incubated for 30 minutes at room temperature. The growing media was removed from the cultured cells and kept at 37°C. Warmed fresh growing media was gently added to the cells and the Lipofectamine2000 solution containing DNA was added drop-wise to the dish. The cells were incubated at 37°C for 1-hour. Subsequently, the transfection media was removed and the cells were gently washed twice in warm PBS. The original growing media was added back to the cells and they were incubated at 37°C for 2-hours to enable eGFP expression. eGFP expression was detected in live cells by epifluorescence.

- *Transfection by electroporation*

The Mouse Neuron Nucleofector® Kit (Amaxa Biosystems) was used to transfect dissociated primary neurons with VAMP2-mRFP for time-lapse imaging according to the manufacturers instructions. In brief, 2.5 x 10^6 freshly dissociated primary rat hippocampal neurons were prepared as described above. When the neurons were in single cell suspension the required amount of cells were transferred to an eppendorf and centrifuged at 1000 RPM for 5-minutes. The supernatant was removed and the cells were resuspended in 100 µL of Nucleofector reagent (Amaxa Biosystems). 3 µg of VAMP2-mRFP DNA construct was added to the resuspended cells and mixed thoroughly by carefully pipetting the solution up and down. The mixture was transferred to an Amaxa cuvette and placed into the Nucleofector machine. The G-13 program was used to transfect the neurons. Once the program was complete, the transfected cells were added to previously conditioned warm plating medium and plated at the density required. The primary neurons were incubated as described above and the plating solution was exchanged with warm growing solution after 3-hours.

2.5. Protein and drug treatments

Wnt7a (50 ng/mL) (R and D systems) **and Dkk1** (20 ng/mL) (R and D systems), prepared in 0.1% BSA, were used to activate or inhibit Wnt signaling respectively in hippocampal cultures. Unless otherwise stated, primary hippocampal neurons were treated with Wnt7a overnight, and

with Dkk1 for 2-hours. Wnt7a and Dkk1 were mixed with growing media and warmed to 37°C before treating the cells.

Actinomycin-D (AmD) (Sigma) is an RNA polymerase inhibitor and was used to block transcription. AmD was solubilized in DMSO and used at a final concentration of 10 µg/mL. For the experiment, AmD/vehicle was diluted in warm growing solution. Primary hippocampal neurons were pre-treated in AmD for 30-minutes; this was ensure that transcription was arrested prior to the addition of Wnt7b or Dkk1. For the treatment period, AmD was added to Wnt7b conditioned media or to Dkk1 containing media. The cells were incubated for 2-hours prior to fixation.

BIO (Calbiochem) was used to inhibit Gsk3 activity to mimic canonical-Wnt signaling (Meijer et al., 2003; Tseng et al., 2006). A 1 µM solution of BIO was prepared by solubilizing in DMSO. DMSO was used as the control. BIO or DMSO were used alone and in conjunction with Dkk1, where it was mixed with the Dkk1 containing growing media prior to adding it to the cells.

2.6. Preparation of plasmid DNA

VAMP2-mRFP expression construct was a kind gift from Dr Matthijs Verhage. VAMP2-mRFP was amplified using the Qiagen EndoFree® Plasmid Maxi Kit according to manufacturer's instructions. Briefly, a sterile pipette tip was used to scrape an aliquot of *E.coli* containing the VAMP2-mRFP vector stored as glycerol stock at -80°C. The pipette tip was placed directly in a round-bottomed tube containing 5mL of Luria-Bertani (LB) broth supplied with kanamycin antibiotic at 50ug/mL final concentration. The solution was incubated overnight at 37°C with vigorous shaking (220 rpm) to grow a bacterial starter culture. 1mL of the starter culture was then used to inoculate 200mL of LB broth containing kanamycin and the culture was further incubated for 16 hours with vigorous shaking (220 rpm). Bacteria were harvested by centrifuging at 6000 x g for 15 minutes at 4°C. The pelleted bacteria were re-suspended in the appropriate buffer prior to the alkaline lysis reaction (all buffers and reagents provided in the Qiagen Maxi Kit). The cell lysate was then filtered and washed and the DNA was eluted. DNA was subsequently precipitated using 0.7 volumes of isopropanol with centrifugation at 15,000 x g for 30 minutes at 4°C. The DNA pellet was washed with 70% ethanol and air-dried before being dissolved in endotoxin-free TE buffer (Qiagen). DNA yield was determined by absorbance readings at 260nm using a spectrophotometer.

2.7. Immunohistochemistry

- *Dissociated cultures*

Fixation: Primary neurons cultured on glass coverslips were gently washed twice in warm PBS prior to fixation in either warm 4% paraformaldehyde (PFA) at room temperature for 18 minutes, or in ice-cold methanol at -20°C for 4 minutes. Methanol fixation was used for PSD95 labeling.

Particular care was taken when aspirating and adding solutions to minimize the possibility of damage to the cells. **Permeabilization:** Neurons were permeabilized with 0.02% Triton in PBS for 2-minutes at room temperature, then washed 3 times in PBS. **Staining:** Neurons were blocked for 1-hour at room temperature in 5% BSA in PBS. Incubation in primary antibodies, diluted in 1% BSA was for either 1-hour at room temperature or overnight at 4°C. Samples were then washed 3 times in PBS over 15-minutes before application of fluorescence-conjugated secondary antibody diluted in 1% BSA for 1-hour at room temperature in the dark. The coverslips where again washed 3 times in PBS over 15-minutes before mounting on glass slides using Fluoromount-G (Southern Biotech). See section 2.10 for a full list of antibodies used. The coverslips were left overnight and in the dark at room temperature to allow the mounting medium to harden, then stored in the dark at -20°C to optimize fluorescence preservation.

- *TUNEL and Hoechst staining*

Terminal deoxynucleotidyltransferase-Mediated dUTP-Digoxygenin nick end labeling (TUNEL) was used to detect neuronal apoptosis. The Apoptag® red *in situ* apoptosis detection kit (Chemicon) was used following manufacturer's instructions. All buffer solutions were supplied as part of the kit. Briefly, primary hippocampal neurons were fixed and permeabilized in ice cold EtOH/HAc (2:1) for 30 minutes at -20°C. Samples were then washed in PBS 3 times for 2-minutes per wash. 30 µL of Equilibrium buffer was pipetted onto each coverslip and left for 1-minute. The terminal deoxynucleotidyl transferase (Tdt) reaction runs for 1-hour at room temperature. Coverslips were washed in Stop/Wash buffer for 10-minutes with agitation, then washed 3 times in PBS. Secondary antibodies were applied for 1-hour at room temperature, then washed 4 times over 8-minutes.

Hoechst stains are bis-benzimides that fluorescently label nuclei. They were used in conjunction with the TUNEL assay to provide a count for the total number of nuclei. Following secondary antibody incubation and PBS washes in the TUNEL protocol, 2.5 µg/mL (diluted in PBS) bis-benzimide (Sigma) was added to the cover slips for 15-minutes. The coverslips were then washed 3 times is PBS and mounted on glass slides using Fluoromount-G (Southern Biotech).

2.8. FM1-43 synaptic vesicle re-cycling

This approach exploits the complex system of SV recycling, which is stimulated by neural activity. In summary, following stimulation with KCl, docked SVs fuse with the plasma membrane to release their neurotransmitter contents into the synaptic cleft. To compensate for this addition of plasma membrane, vesicles are retrieved from the plasma membrane either by budding off or by clathrin-dependent endocytosis (Figure 5.2 panel A). FM1-43 (Molecular Probes) is a fluorescent amphipathic styryl dye that labels the plasma membrane and is taken up by retrieved SVs during SV recycling (Gaffield and Betz, 2006).

Primary hippocampal cells were cultured to 14 DIV. Growing solution was removed and neurons were gently washed once with warm KRH buffer. Warm depolarization buffer (55mM KCl in KRH buffer) was added to the cells for 90 seconds. The samples where washed three times for 20-seconds per wash in warm KRH buffer. The samples where then fixed with warm 4% PFA for 18 minutes before washing in PBS and mounting onto glass slides with Fluoromount-G (Southern Biotech).

2.9. Fixation for electronmicroscopy

Primary hippocampal neurons were prepared and plated at medium-high density, and then cultured for 21 days. Primary fixation was in warm 2% PFA with glutaraldehyde for 1-hour. Samples were subsequently washed in 0.1M cacodylate buffer twice for 5-minutes each. For post fixation, the samples were incubated in 1% osmium tetroxide in 0.1M cacodylate buffer ph7.3 for 1-hour at 4°C, then washed in 0.1M cacodylate buffer for 5-minutes. The cells were further washed in dH_2O for 5-minutes before 20-minutes incubation in 0.5% uranyl acetate in dH_2O at room temperature. The specimens were dehydrated by serial ethanol washes of 5-minutes each before mounting in resin. Progressive ratios of propylene oxide and resin mix were used to ensure complete penetration of the resin mix, the cells were left for 1-hour in each mix, then the final 100% resin mix was overnight. Coverslips were mounted onto a beem capsule filled with 100% resin and left for 24-hours at 60°C to harden. Mark Turmaine, the senior technician at our Departmental EM facility, further processed the samples by preparing 70 nm slices from the specimens and mounting them onto 300 mesh copper grids. Only samples from basal slices were used for imaging. EM images were acquired using a JEOL1010 electron microscope at a magnification of x 60,000. The acquired images were analyzed using Metamorph software (Molecular Devices). For quantifications, the active zone and postsynaptic density were traced manually.

2.10. Fluorescence imaging and data analysis

- *Fixed tissue samples*

Epifluorescence microscopy was used to analyze individual synaptic puncta and FMI-43 staining. Images for these experiments were captured using either an Olympus BX60 upright microscope, or an Axiovert Zeiss 200 inverted microscope. Images for analysis were acquired with either a 100x or 63x oil immersion objective. The numerical aperture of the Olympus BX60 100x objective was 1.30 and the pixel dimension obtained 64.5 nm^2. The numerical aperture of the Axiovert Zeiss 200 63x objective was 1.40 and the pixel dimension obtained 25 nm^2. A total of 14-16 fields of view were acquired from 2 separate coverslips from each condition per experiment. All imaging experiments were in triplicate from independent experiments using different cultures. Images were analyzed using Metamorph software (Molecular Devices) to determine synaptic puncta number, size and intensity, or Kontron KS400 (Image Associates) for

neurite area. In all cases, the presence, intensity and size of synaptic puncta or Tuj-1 labeled neurites were determined using intensity thresholds. Minimum and maximum intensity thesholds for each experiment were established visually from control conditions. Minimum thresholds were set to exclude low-level fluorescing objects not representative of synaptic protein clusters typically found at synaptic sites; maximum thesholds were set to resolve neighboring fluorescent puncta that would otherwise appear merged together as a single large object. Intensity thresholds were sampled across 4-5 images before appropriate settings were finally determined. Intensity thresholds were set for each spectral channel for each experiment. Once a threshold and exposure time had been selected for each channel, the exact parameters were applied for each image in the experiment. For the majority of puncta size analysis experiments, thresholded objects smaller than 0.1 μm^2 and larger than 1.5 μm^2 where rejected. This protocol was not applied to the analysis of puncta size distribuion were no upper or lower limits were applied. The data generated from each experiment was logged into Excel for statistical analysis.

Images of co-localized synaptic puncta were captured using a Leica TCS SP1 lazer scanning confocal microscope. Images for analysis were acquired with a 63x oil immersion objective. The numerical aperture of the Leica TCS SP1 objective was 1.32 and the pixel dimension obtained was 155 nm^2. A total of 12-14 fields of view were acquired from 2 separate coverslips from each condition per experiment. 8-10 frame stacks were acquired in the z-plane spanning the depth of the neurites, which were grown as a monolayer. All imaging experiments were in triplicate from independent experiments from different cultures. Images from confocal microscopy were analyzed using Velocity (Improvision). Intensity thresholds were applied as described above. Co-localization analyses were performed using a customized protocol set within Velocity. In summary, objects of interest (puncta) were identified by intensity; objects less than 0.1 μm^3 and larger than 1.5 μm^3 were excluded; an object size guide was entered (0.3 μm^3). This information was entered for each synaptic marker being assayed. The protocol excluded non-touching objects for the two markers; therefore only objects that were touching each other were selected for analysis. The data generated from each experiment was logged into Excel for statistical analysis.

- *Time-lapse*

Time-lapse experiments were performed on a Leica TCS SP1 confocal microscope fitted with a heated stage and CO_2 chamber. To minimize focus drift, the stage was heated for at least 4-hours prior to recoding. Images for analysis were acquired with a 63x water-dipping objective. The numerical aperture of the Leica TCS SP1 objective was 0.9 and the pixel dimension obtained 430 nm^2. 8-10 z-frame stacks were acquired every 5-minutes. In preliminary experiments, images were acquired every minute, however to optimize fluorescence 5-minute intervals were subsequently used. To add the control (0.1% BSA) or Dkk1 containing media, the time-lapse was paused, and 500 uL of the growing media was carefully removed from the culture dish whilst it was still mounted in the heated chamber; the same volume of new media was added to the chamber, and the time-lapse recording was resumed. The acquired images

were analyzed using Metamorph software (Molecular Devices). Intensity thresholds were applied as described above. The data was logged into Excel for statistical analysis.

2.11. Statistical analysis

Normality of data was determined using the Shapiro-Wilks test on control data. Due to limitations of handling large data sizes (maximum 2000 data points), data points for the Shapiro-Wilks test were randomly selected using the Excel function. Sampled data generated from control puncta analysis experiments fell within a normal distribution. In all cases, comparisons for statistical analysis were made against two conditions (control and treated cells) using the full data set. ANOVA single-factor was used to determine statistical significance. Statistical significance is indicated in the text and figures as follows: * $p<0.05$, ** $p<0.005$, *** $p<0.001$.

2.12. Antibodies

Primary antibodies	Species	Supplier	Dilution	
			IHC*	WB**
Bassoon	Mouse	Bioquote	1:500	
Cask	Mouse	Chemicon	1:500	1:1,000
ERK	Rabbit	Santa Cruz		1:2,000
Gephyrin	Mouse	Synaptic Systems	1:500	
HA-tag	Rat	Roche	1:500	
Neuroligin2	Goat	Santa Cruz	1:100	
PSD-95	Mouse	Affinity Bioreagents	1:200	1:770
Synapsin1	Mouse	Abcam	1:700	1:2,500
Tuj1	Mouse/Rat Chick	Chemicon	1:500	
Tyrosinated tubulin (Y1/2)	Rat	Oxford Biotechnology	1:200	
VAMP2	Mouse	Synaptic Systems	1:2,000	1:10,000
VAMP2	Rabbit	Synaptic Systems	1:1,500	
vGat	Rabbit	Synaptic Systems	1:1,000	
vGlut	Guinea pig	Chemicon	1:5,000	

* IHC: Immunohistochemistry. ** WB: Western blot

Secondary antibodies	Conjugate	Supplier	Dilution
Goat anti-gunea pig IgG	Alexa 488	Molecular Probes	1:600
Goat anti-gunea pig IgG	Alexa 568	Molecular Probes	1:600
Donkey anti-chicken IgG	Dy-Light 488	Jackson Immunoreseach	1:600
Donkey anti-rabbit IgG	Alexa 594	Molecular Probes	1:600
Donkey anti-mouse IgG	Alexa 488	Molecular Probes	1:600
Goat anti-mouse IgG	Alexa 568	Molecular Probes	1:600
Goat anti-rat IgG	Alexa 647	Molecular Probes	1:600
Donkey anti-goat	Alexa 568	Molecular Probes	1:600
Sheep anti-mouse IgG	HRP	Amersham	1:2500
Donkey anti-rabbit	HRP	Amersham	1:2500

2.13. Solutions

Solutions for preparing and fixing cultured cells

- **Hippocampal plating medium (50 mL)**

D-MEM (Gibco)	45 mL
Horse serum (Gibco)	5 mL
Penicillin/streptomycin (20 µg/mL) (Gibco)	200 µL
1 mM Sodium pyruvate (Sigma)	5.5 mg
2 mM L-glutamine (Sigma)	14.5 mg

- **Hippocampal growing medium (50 mL)**

Neurobasal medium (Gibco)	48.5 mL
B27 supplement (Invitrogen)	1 mL
N2 supplement (Invitrogen)	0.5 mL
15 mM Ovalbumin (Sigma)	50 mg
1 mM Sodium pyruvate (Sigma)	5.5 mg
2 mM L-glutamine (Sigma)	14.5 mg

- **Borate Buffer (400mL)**

Sterile filtered water	400 mL
50 mM boric acid (Sigma)	1.24 g
25 mM borax (Sigma)	1.9 g
pH to 8.5	

- **Growth media for conditioned media (50 mL)**

DMEM-Glutamax (Gibco)	45 mL
10% Foetal calf serum (Gibco)	5 mL
Penicillin/streptomycin (final 20 µg/mL, Gibco)	200 µL
0.4 mM Geneticin G418 sulphate (Sigma)	70 µL

- **Phosphate buffered solution (1 L of 10x PBS)**

1.4 M NaCl	80 g
26 mM KCl	2 g
15 mM KH_2HPO_4 (anhydrous)	2 g
14 mM Na_2HPO_4 (anhydrous)	2 g
pH the 1x slution to 7.5	

- **4% Paraformaldhyde (50 mL)**

1.3 M paraformaldhyde (BDH)	2 g
0.1 M sucrose (Sigma)	2 g
0.1 mM NaOH (BDH)	200 µg

2 x PBS	25 mL
dH_2O	25 mL

Solutions for Western blot

• **Resolving gel** (20 mL sufficient for 2 x 0.5 mm mini gels)	**10%**	**15%**
dH_2O	7.9 mL	4.6 mL
Acrylamide (Sigma)	6.7 mL (1.5 mM)	10.0 mL (2.3 mM)
1.5 M Tris (pH 8.8)	5.0 mL	5.0 mL
1.7 mM Sodium dodecyl sulphate (SDS) (Sigma)	0.2 mg	0.2 mg
2 mM Ammonium persulphate (Sigma)	0.2 mg	0.2 mg
TMED (Sigma)	0.008 mL	0.008 mL

• **5% Stacking gel** (10 mL sufficient for 2 x 0.5 mm mini gels)	
dH_2O	6.8 mL
0.8 mM Acrylamide (Sigma)	1.7 mL
1.0 M Tris (pH 6.8) (Sigma)	1.2 mg
3.5 mM Sodium dodecyl sulphate (SDS) (Sigma)	0.1 mg
4.4 mM Ammonium persulphate (Sigma)	0.1 mg
TMED (Sigma)	0.01 mL

• **Tris buffered solution (TBS)**	
1 M Tris (ph 7.5) (Sigma)	10 mL
5 M NaCl	30 mg
Made up to 1L with dH_2O	

- **TBST** (TBS with 0.1% Tween)
  1 mL of Tween 20 per litre of TBS (Sigma)

• **Running buffer**	
Tris/glycine solution	100 mL
0.35 mM Sodium dodecyl sulphate (SDS) (Sigma)	100 mg
Topped up to 1 L with dH_2O	

• **Blottiing buffer**	
Tris/glycine solution	100 mL
MeOH	200mL
0.35 mM Sodium dodecyl sulphate (SDS) (Sigma)	2 mg
Topped up to 1 L with dH_2O	

• **Tris 10x (1 L)**	
Tris (Sigma)	30.3 g

2 mM Glycine (Sigma)	144 g
dH_2O	1 L

- **Protein loading buffer (10 mL)**

1.4 mM Sodium dodecyl sulphate (SDS) (Sigma)	4 mg
4.3 mM Glycerol (Sigma)	4 mL
1M Tris (ph 6.8)	1.2 mg
0.05 mM B-mercaptoethanol (Sigma)	36 µL

- **RIPA buffer (50 mL)**

50mM Tris (pH 8)	2.5 mL
150 mM NaCl	0.438 g
20% Triton (Sigma)	2.5 mL
0.01 mM Sodium deoxycholate (Sigma)	0.25 g
0.03 mM Sodium dodecyl sulphate (SDS) (Sigma)	0.5 mg
dH_2O	45 mL

Solutions for FM1-43 recycling assay

- **KRH buffer (50 mL)**

5 M NaCl	1.25 mL
0.5 M HEPES (Signa)	2.5 mL
1 M KCl	0.25 mL
100 mM $CaCl_2$	1 mL
100 mM $MgSO_4$	0.6 mL
6 mM Glucose	54 mg
1.2 mM KH_2PO_4	8.1 mg

- **Depolarization buffer (55 mM KCl)**

1M KCl	110 µL
0.6 mM Aminophosphonovaleric acid (APV) (Ascent Scientific)	20 µL
0.5 mM 6-cyano-7-nitroquinoxaline-2,3-dione(CNQX) (Ascent Scientific)	20 µL
0.2 mM FM1-43 (Molecular Probes)	20 µL
KRH buffer	2mL

Solutions for EM fixation

- **2% paraformaldehyde (100 mL)**

0.6 M Paraformaldehyde	2 mg
0.6 M Glutaraldehyde	6 mL
0.1 M Cacodylate buffer (sodium cacodylate in dH_2O)	50 mL
dH_2O	44 mL
pH to 7.3	

- **Agar resin mix**

Agar 100 (Agar Scientific)	12 g
Dodecenyl Succinic Anhydride (DDSA) (Agar Scientific)	8 g
Methylnadic anhydride (MNA) (Agar Scientific)	5 g
Benzyldimethylamine (BDMA) (Agar Scientific)	16 drops

Solutions for preparing plasmid DNA

- Luria Bertani (LB) broth

  LB supplied in powder form (Invitrogen)

  20 g per litre of dH_2O

CHAPTER 3:

3. Results: Wnt7a selectively stimulates the assembly of excitatory synapses in hippocampal neurons

3.1. Introduction

The formation of functional neural circuits depends critically on the assembly of precise synaptic contacts between neurons and their target cells. In addition, the specification of synapse type is equally important in the formation and maintenance of neural circuits. Central synapses are broadly categorised as being either excitatory or inhibitory, and the appropriate balance between these two types of synapses is essential for proper function. Abnormalities in the formation of either excitatory or inhibitory synapses, or the failure to establish the proper balance during early development is associated with a number of neurodevelopmental disorders that include mental retardation (Gao, 2002; Gibson et al., 2008; Pan et al., 2004), Schizophrenia (Angelucci et al., 2005; Walsh et al., 2008) and autism (Feng et al., 2006; Sudhof, 2008). Importantly, synaptogenesis is a process that occurs throughout life. Indeed, it is believed that the formation of new synapses is critical for the remodeling of neural circuits in an experience dependent manner and may be a biological basis for life-long learning and memory (Bailey et al., 2004; Eaton and Davis, 2003; Holtmaat and Svoboda, 2009; Waites et al., 2005). Thus, elucidating the mechanisms underlying synaptogenesis may yield critical insights into the processes of neurodegenerative disorders, as well as ongoing neural activity in the healthy brain.

Synapse formation requires the recruitment and assembly of thousands of proteins at a single point of contact between a pre- and postsynaptic partner (Emes et al., 2008). A number of models describe the initial formation of synaptic contacts and recruitment of synaptic proteins (Juttner and Rathjen, 2005; McAllister, 2007; Waites et al., 2005) yet the molecular mechanisms that regulate synaptic assembly remain poorly understood. All of the proposed models require the establishment of a molecular dialogue between the pre and postsynaptic elements. Membrane bound molecules such as SynCam (Biederer et al., 2002; Ko et al., 2006) and transynaptic complexes of neuroligin/neurexin, Eph/ephrin and cadherin/catenin have all been described as key regulators of synaptogenesis once initial contact has been made between pre- and postsynaptic partners (McAllister, 2007; Waites et al., 2005). Only in recent years has the search for specific molecular regulators of excitatory versus inhibitory synapses been addressed. Compelling evidence now exists for specific neuroligin/neurexin isoforms as determinants of either excitatory or inhibitory synapses (Chih et al., 2005; Chubykin et al., 2007), whereas SynCam specifically instructs the formation of excitatory synaptic components (Biederer et al., 2002). Whilst the role of these membrane-bound molecules is being

unravelled, the upstream mechanisms that recruit them to the nascent synapse remains poorly understood and whether secreted factors participate in this process is also unknown.

Overwhelming evidence supports a role for secreted factors as primary instigators of synapse assembly. Molecules such as BDNF (Alsina et al., 2001; Baldelli et al., 2002; Cunha et al.; Lu et al., 2009; Sanchez et al., 2006; Sato et al., 2007), FGFs (D'Sa et al., 2007; Fox and Umemori, 2006; Umemori et al., 2004) and Wnts (Ahmad-Annuar et al., 2006; Davis et al., 2008; Hall et al., 2000; Lucas and Salinas, 1997) have all been characterised as potent synaptogenic factors. Both BDNF and FGFs stimulate the formation of excitatory and inhibitory synapses in different regions of the brain and can be described as general synaptic organisers. Wnt proteins have been shown to act as target-derived signals in the cerebellum that regulate presynaptic remodelling and stimulate protein assembly (Ahmad-Annuar et al., 2006; Hall et al., 2000; Lucas and Salinas, 1997). This role at excitatory mossy fibre synapses was established by both loss and gain-of-function analyses in vertebrate systems. In the murine hippocampus, Wnts stimulate both pre- and postsynaptic differentiation (Ahmad-Annuar et al., 2006; Ciani et al., 2011; Davis et al., 2008; Farias et al., 2009), and a role for Wnts as postsynaptic organisers in the peripheral nervous system has also been demonstrated during fly and chick development (Ataman et al., 2008; Henriquez et al., 2008; Packard et al., 2002). However a clear distinction of whether Wnts preferentially stimulate the formation of excitatory or inhibitory synapses had not been previously addressed when I began this investigation. Wnt7a and Wnt7b are strongly expressed in the hippocampus throughout early postnatal development, and into adulthood (Davis et al., 2008; Rosso et al., 2005; Shimogori et al., 2004). The expression of Wnt7a/b in the adult brain is particularly intriguing as it could suggest an ongoing role for Wnt signalling in modulating synaptogenesis and/or synapse function in the adult brain.

In this chapter I further examine the role of Wnt-mediated synaptogenesis by using immunohistochemistry and ask whether Wnt signalling specifically stimulates the formation of excitatory synapses. My results show that exogenous Wnt7a increases the number of synaptic puncta for a variety of pre and postsynaptic markers and importantly, increases the incidence of overlap between synaptic vesicles (SVs) and active zone (AZ) proteins indicative of presynaptic assembly. Crucially, Wnt7a specifically stimulates the clustering and alignment of excitatory pre- and postsynaptic markers without affecting the distribution of inhibitory markers. These data support the hypothesis that Wnt signalling promotes full synapse assembly and importantly, separates Wnts from other secreted pan-synaptogenic factors as specifically stimulating excitatory synapse formation. These findings were published during the final preparation of this thesis manuscript (Ciani et al., 2011).

3.2. Results

3.2.1.Wnt signalling stimulates presynaptic assembly and postsynaptic differentiation

Immunohistochemical evidence from our lab, and others, has revealed an increase in the number of synaptic protein clusters in response to exogenous Wnt7a/b (Ahmad-Annuar et al., 2006; Davis et al., 2008; Farias et al., 2009; Hall et al., 2000; Lucas and Salinas, 1997). To further examine the role of Wnts as synaptogenic factors I tested whether Wnt7a could induce the assembly of multiple synaptic proteins to single loci. These data would determine whether Wnts increase the number of assembled synapses rather than just increasing the number of synaptic protein clusters in transit (Ahmari et al., 2000; Lee et al., 2008b; Sabo et al., 2006; Shapira et al., 2003; Tao-Cheng, 2007). The protein markers for this assay were selected for their functional diversity; VAMP2 is a presynaptic synaptic vesicle (SV) associated protein, Bassoon is a presynaptic cytomatrix protein that forms part of the active zone and PSD-95 is a postsynaptic scaffold protein. Dissociated hippocampal cultures were grown at low to medium density (50-100 cells/mm^2) and treated at 14 DIV. The peak of synaptogenesis in hippocampal cultures is believed to occur at approximately 14 DIV (Fletcher et al., 1991). This stage of development was chosen as the system is primed for synapse assembly and most pre and postsynaptic markers can be clearly visualized. Cultures were treated overnight with Wnt7a (50ng/mL) or vehicle (0.1% BSA) by bath application before fixation with either 4% PFA or cold methanol and processing for immunohistochemistry. Samples were analysed by confocal microscopy.

Exogenous Wnt7a significantly increased the number of presynaptic puncta (Fig.3.1 panels A and B); VAMP2 puncta were increased by 29% (Fig.3.1 panel C, Control 45.5 p/na, ±2.5, Wnt7a 58.9 p/na, ±5.9, p=0.024) and Bassoon puncta were increased by 38% (Fig.3.1 panel C, control 46.5 p/na, ±3.39, Wnt7a 64.0 p/na, ±6.82, p=0.014,). Similarly on the postsynaptic side, exogenous Wnt7a increased the number of PSD-95 puncta by 34% (Fig.3.2 panel C, control 45.9 p/na, ±2.9, Wnt7a 61.7 p/na, ±4.3, p=0.003). The parallel increase for the postsynaptic marker, PSD-95, was especially encouraging as a role for Wnt7a in postsynaptic differentiation had not been previously demonstrated. These data suggest that Wnt7a stimulates both pre and postsynaptic differentiation in young hippocampal cultures.

To determine whether Wnt7a was increasing the number of synaptic sites, rather than just the number of presynaptic puncta in transit, co-localisation analysis was performed. The control data shows that under basal conditions 61% (±2.7) of VAMP2 co-localised with Bassoon; the remaining 39% are likely to represent SVs in transit (Fig.3.1 panel E). Similarly, 64% (±1.4) of the total Bassoon puncta population co-localised with VAMP2 puncta; the remaining 36% of Bassoon puncta are also likely to be protein clusters in transit (data not shown). Importantly, following Wnt7a treatment the number of VAMP2 puncta co-localised with Bassoon increased significantly by 31% (Fig.3.1 panel D, control 27.5 p/na, ±1.54, Wnt7a 35.9 p/na, ±3.90, p=0.029). Similarly, the number of Bassoon puncta co-localised with VAMP2 increases

significantly by 38% (Fig.3.2 panel D, control 28.9 p/na, ±1.59, Wnt7a 39.9 p/na, ±4.46, p=0.012). Intriguingly, when quantifying the percent of total VAMP2 puncta co-localised with Bassoon, there is no significant change between control and Wnt7a treated neurons (control 61.1% ± 2.7, Wnt7a 63.3% ± 2.7, Fig.3.1. panel E). Similarly, there is no significant change in the percentage of total Bassoon puncta co-localising with VAMP2 puncta (data not shown). In summary, these data demonstrate that Wnt stimulates the assembly of functionally diverse presynaptic proteins to single loci, and thus strongly indicates the assembly of nascent synapses.

3.2.2.Wnt signalling specifically regulates excitatory synapse formation

I next examined whether Wnt7a has pan-synaptogenic activity or whether it has a preferential effect on excitatory synapses. To address this issue I examined the effect of Wnt7a on the level and localization of pre and postsynaptic markers for either excitatory or inhibitory synapses using confocal microscopy. The presynaptic markers used were vGlut and vGat, which are vesicle-associated proteins that transport glutamate or GABA from the cytoplasm into synaptic vesicles; these are excitatory and inhibitory neurotransmitters respectively. For postsynaptic labelling, the scaffold proteins PSD-95 and Gephyrin were used to label excitatory and inhibitory synapses respectively. 14 DIV Cultures were treated overnight with Wnt7a (50ng/mL) or vehicle (0.1% BSA) before fixation with either 4% PFA or cold methanol and processing for immunohistochemistry. Samples were analysed by confocal microscopy.

Results show clear disparity in the type of synapse that Wnt regulates; in response to overnight Wnt7a treatment the number of vGlut and PSD-95 puncta were significantly increased (Fig.3.2. panel C, vGlut 28% increase, control 27.6 ±1.6, Wnt7a 35.3 ±1.5, p=0.001. PSD-95 34% increase, control 45.9 p/nl ±2.9, Wnt7a 61.7 ±4.4, p=0.003). In contrast, the number of vGat and Gephyrin did not significantly change (Fig.3.2. panel E, vGat 0.002% increase, control 8.1 ±0.87, Wnt7a 8.1 ±0.64. Gephyrin 0.05% decrease, control 7.1 p/nl ±1.2, Wnt7a 6.8 ±1.2). I next examined the effect of Wnt7a on the colocalization of pre and postsynaptic markers to evaluate the number of assembled synaptic sites. Wnt7a significantly increased the proportion of PSD-95 puncta that co-localises with vGlut puncta (34% increase, Figure 3.2 panel D, control 41.2% ± 3.3, Wnt7a 55.6% ± 3.7, p=0.008). In contrast, the levels of co-localisation between vGat and Gephyrin did not significantly change (Figure 3.2 panel F, control 29.9% ±2.1, Wnt7a 36.4% ±2.1). Together these results provide compelling evidence that Wnt7a selectively stimulates the formation of excitatory synapses and is not a general synaptogenic factor. Furthermore, these data demonstrate that Wnt7a stimulates the alignment of both pre- and postsynaptic partners to form assembled synaptic sites.

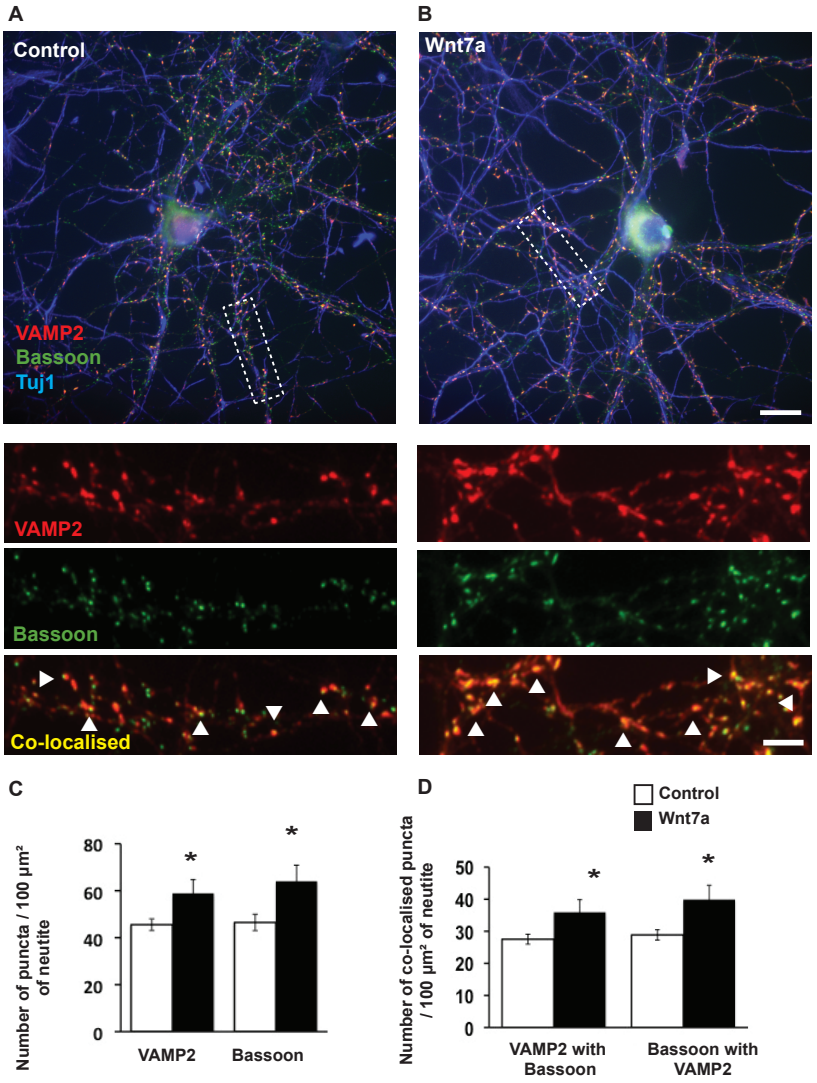


Figure 3.1. Wnt7a induces presynaptic assembly
(A) Cultured hippocampal neurons are immunolabeled to show sites of co-localization between presynaptic proteins. Lower panels are from the region outlined by the white box. (B) Exogenous Wnt7a increases the number of VAMP2 puncta (red) and Bassoon puncta (green) as well as the number of co-localized puncta (yellow). (C) Quantifications show that Wnt7a increases the number of VAMP2 puncta by 29% (p=0.02) and Bassoon puncta by 38% (p=0.01). (D) Wnta7a increases the number of VAMP2 puncta co-localized with Bassoon puncta by 31% (p=0.03) and the number of Bassoon puncta co-localized with VAMP2 puncta by 38% (p=0.01). (E) Quantification reveals no change in the overall percentage of co-localization between VAMP2 and Bassoon. Scale bars; upper panels 10 µm, lower panels 5 µm. *p<0.05, **p<0.005, ***p<0.001 by ANOVA. Error bars represent SEM.

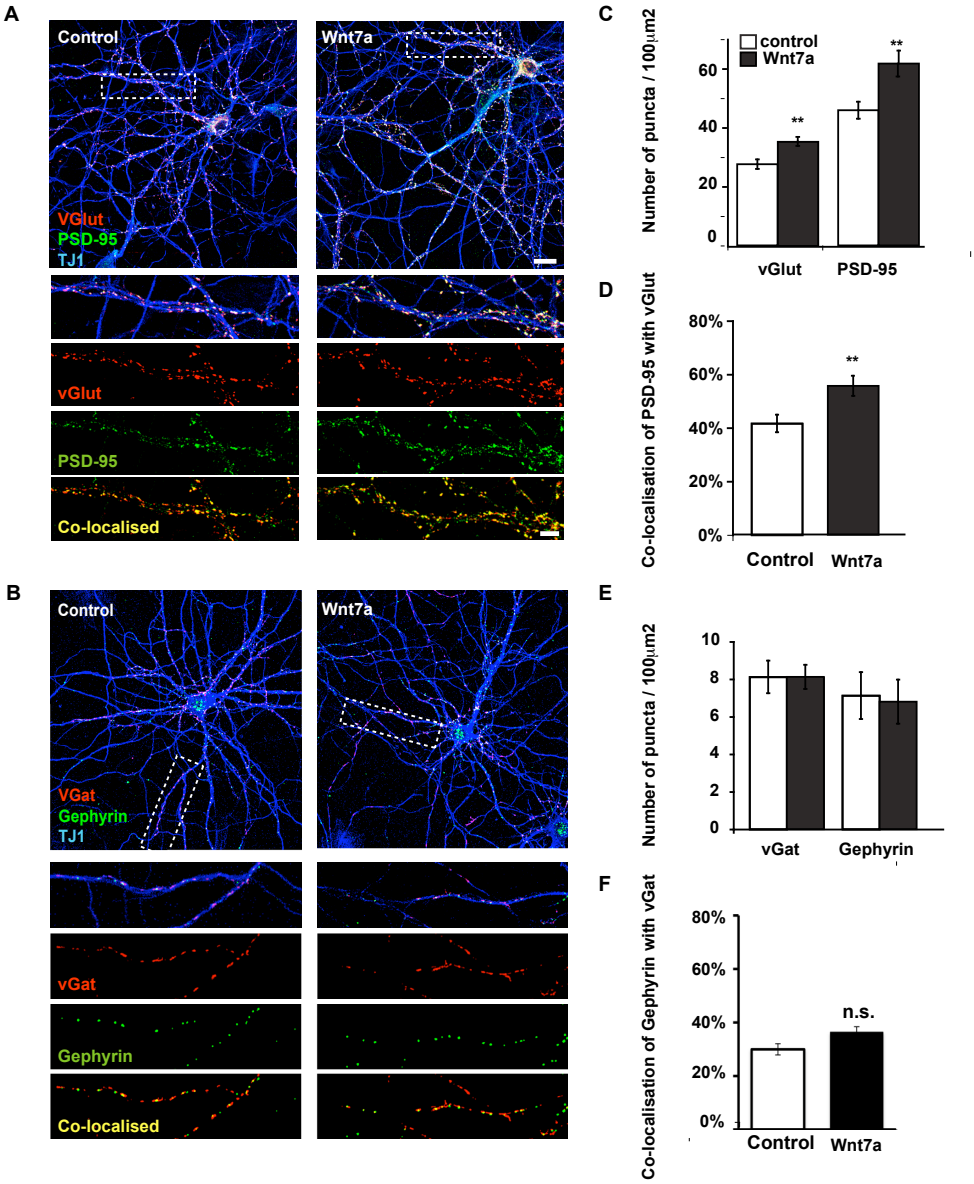


Figure 3.2. Wnt7a specifically stimulates the formation of excitatory synapses in hippocampal neurons. (A) Low and high magnification images show that Wnt7a increases the number of excitatory pre and postsynaptic sites (vGlut1 and PSD-95 respectively). In contrast, (B) Wnt7a does not increase the number of inhibitory pre and postsynaptic sites (vGat and Gephyrin respectively). (C) Quantification reveals a significant increase in the number of both pre- and post-synaptic excitatory puncta in response to Wnt7a treatment (vGlut 30% increase, p=0.001 and PSD-95 increase 34% p=0.003). (D) The percentage of total PSD-95 that co-localizes with vGlut also increases following Wnt7a treatment (34% p=0.007). (E) and (F) Quantification reveals no change in the number of pre- and postsynaptic inhibitory puncta or their levels of co-localization in response to Wnt7a treatment. Scale bars: upper panels = 10 µm, lower panels = 5µm. **p<0.01 by ANOVA. Error bars represent SEM.

3.3. Discussion

Previous work from our lab and others has demonstrated that Wnt7a/b are synaptogenic factors. In particular Wnt has been shown to act as a presynaptic organizer in the vertebrate C (Ahmad-Annuar et al., 2006; Davis et al., 2008; Farias et al., 2009; Hall et al., 2000; Lucas and Salinas, 1997). The analysis of puncta distribution presented here corroborates these data and also explores presynaptic assembly of SVs with active zone material.

Wnt7a increased the number of SV clusters and active zone components along the neurite. Importantly, this correlated with a significant increase in the number of co-localised sites, indicating the assembly of SV pools with the active zones. In both the control and Wnt treated neurons ~62% of the total SV population is co-localized with Bassoon, these data confirm that VAMP2 and Bassoon are not simultaneously transported (Zhai et al., 2001), as in the case of Piccolo and Bassoon (Shapira et al., 2003), and thus make them suitable candidates for studying the assembly of presynaptic components. It is important to note there is no significant change in the total percentage of co-localised puncta between the control and Wnt7a cells; this finding suggests that the newly formed VAMP2 and Bassoon puncta have co-localised. If the newly formed puncta did not assemble to the same loci, then there would be an overall reduction in the percentage of co-localised puncta. In conclusion, this study demonstrates that Wnt7a stimulates the aggregation and coalescence of critical presynaptic components to form nascent synaptic sites.

Wnt7a stimulates postsynaptic differentiation and the alignment of pre- and postsynaptic partners. The results demonstrate that overnight bath application of Wnt7a significantly increases the number of PSD-95 puncta per neurite area suggesting the assembly of postsynaptic apparatus. Importantly, there is a significant increase in the percentage of PSD-95 that co-localises with the presynaptic marker vGlut suggesting the formation of *de novo* synapses. Thus, Wnt7a stimulates the recruitment of PSD-95 to form excitatory postsynaptic sites. These data suggest that Wnt7a signalling does not only stimulate presynaptic assembly but that it also affects postsynaptic differentiation.

Wnt7a specifically stimulates the assembly of excitatory synapses. In contrast to excitatory pre and postsynaptic markers, Wnt7a does not affect the number or colocalization of inhibitory markers such as vGat or Gephryn puncta in hippocampal cultures at 14 DIV. The balance between excitatory and inhibitory synapses is critical to normal brain development and function; therefore, understanding the molecular mechanisms that regulate their assembly is of key importance. Membrane-bound proteins and transynaptic complexes, such as neuroligin/neurexin ioforms and SynCAM have been reported as key organizers of synapse assembly that determine synapse identity in terms of their excitatory or inhibitory transmission properties, but only once initial axodendritic contact has been made. Secreted synaptogenic factors, may act upstream to stimulate contact events but little is known about the mechanisms that could link these events. It was previously proposed that secreted factors might act as

“priming” factors to stimulate synapse formation (Craig et al., 2006). However compelling evidence now suggests that secreted factors are instructive agents of synapse assembly, for example time-lapse imaging studies have shown SV proteins cluster and stabilise prior to axodendritic contact (Sabo et al., 2006). Furthermore, work from our lab has shown that Wnts stimulate the recruitment of presynaptic components within 15 minutes (Ahmad-Annuar et al., 2006); such a rapid response is also consistent with an instructive role. Secreted synaptogenic factors such as BDNF and FGFs act as general instigators of synaptic differentiation by stimulating both excitatory and inhibitory synapse formation. The findings presented here contributed to the first report to demonstrate that a secreted factor can specifically stimulate the assembly of excitatory synapses (Ciani et al., 2011).

In summary, the data presented demonstrates that Wnt7a signalling stimulates pre- and postsynaptic differentiation and the correct alignment of pre- and postsynaptic partners. Importantly, this alignment is specific to excitatory synapses, as there are no effects on the formation of inhibitory synapse. These data provide important insights into understanding how excitatory and inhibitory neural circuits are formed. In the following chapter the role of Wnt signalling in presynaptic differentiation is explored, in particular, the governing molecular mechanisms are probed.

CHAPTER 4:

4. A divergent canonical-Wnt signal regulates presynaptic differentiation

4.1. Introduction

Secreted Wnt proteins are critical instigators of synaptogenesis and play essential roles in establishing neuronal circuits in both vertebrate and invertebrate systems (Budnik and Salinas, 2011; Ciani and Salinas, 2005; Salinas and Zou, 2008). Wnt7a/b can stimulate presynaptic differentiation in vertebrates by recruiting synaptic components to nascent sites (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Davis et al., 2008; Hall et al., 2000). Whilst a role for Wnts during synaptogenesis is firmly established, the downstream signaling cascades at vertebrate synapses, and therefore molecular mechanism, have not yet been characterised.

Wnts are capable of initiating many diverse and complex signalling cascades depending on the cellular context (Logan and Nusse, 2004; van Amerongen and Nusse, 2009), these signalling events have been traditionally categorised as either canonical or non-canonical pathways. Work from our lab and others suggest a role for the canonical-Wnt pathway during synaptogenesis (Ahmad-Annuar et al., 2006; Davis et al., 2008; Hall et al., 2000). In traditional canonical-Wnt siganlling, Wnt ligands bind to their Frizzled receptors and the co-receptor LRP5/6 (Tamai et al., 2000). This complex activates the scaffold protein Dishevelled (Dvl), which in turn inhibits the serine-thronine kinase, Gsk3β, via a macromolecular complex known as the “destruction complex” (Figure 1.4 panel B). Inhibition of Gsk3β enables stable, cytosolic β-catenin to translocate to the nucleus where it binds with the β-catenin responsive transcription factors, TCF and LEF, to initiate transcription (Cadigan, 2008). In the absence of Wnt ligand, Gsk3β phosphorylates β-catenin, which labels it for degradation by the ubiquitin-proteasome (MacDonald et al., 2009). Consequently, β-catenin mediated transcription is inhibited.

Analyses from mutant mice deficient in Wnt signaling reveal significant deficits in synaptic structure and function (Ahmad-Annuar et al., 2006; Hall et al., 2000). Additional reports from our lab have also shown that blockade of Wnt signalling by addition of secreted Frizzled related protein-1 (Sfrp1) blocks presynaptic differentiation induced by exogenous Wnt7a/b (Ahmad-Annuar et al., 2006; Rosso et al., 2005). Sfrps bind to extracellular Wnt ligands to prevent receptor blinding and therefore antagonise all Wnt signalling by a process of sequestration. However effective, this loss of function approach does not indicate the molecular mechanisms required for presynaptic differentiation, as all Wnt signalling pathways are blocked. A role for canonical-Wnt signalling during synaptogenesis has been inferred by pharmacological inhibition of Gsk3, which mimics the Wnt effect of clustering presynaptic proteins during the initial stages of synapse formation (Ahmad-Annuar et al., 2006; Davis et al., 2008; Hall et al., 2000). However, studies from our lab and others demonstrate that initiation of the canonical-Wnt

pathway and inhibition of Gsk3 can illicit neuronal behaviors independent of transcription (Ciani et al., 2004; Korkut et al., 2009; Purro et al., 2008). These findings demonstrate additional diversity within the canonical Wnt-pathway.

To further probe the molecular mechanisms of Wnt-mediated synaptogenesis, I used the specific canonical-Wnt antagonist Dkk1 (Glinka et al., 1998; Niehrs, 1999, 2006). Dkk1 binds specifically to LRP5/6, which is the co-receptor specifically required for canonical-Wnt signaling.(Bafico et al., 2001; Mao et al., 2001). Dkk1/LRP5/6 binding prevents formation of the Wnt/Frizzled/LRP5/6 complex and disrupts canonical-Wnt signaling at the receptor level (Figure 1.5). Here I present data that strongly suggests the presence of an endogenous Wnt activity regulating presynaptic differentiation in cultured hippocampal neurons. I also demonstrate that a divergent canonical-Wnt signaling pathway mediates synaptic vesicle (SV) clustering; the signaling pathway diverges downstream of Gsk3β and is transcription independent. These data provide important insights into the molecular mechanisms of Wnt-mediated presynaptic differentiation and provide a clear, albeit preliminary, characterization of the signalling pathway by which Wnts modulate the formation of presynaptic sites.

4.2. Results

4.2.1. Short-term blockade of canonical-Wnt signalling, by Dkk1, inhibits presynaptic differentiation

Bath application of Wnt7b rapidly increases the number of SV clusters in both cerebellar mossy fibers and hippocampal neurons (Ahmad-Annuar et al., 2006). This effect is mimicked by pharmacological inhibition of Gsk3 (Davis et al., 2008; Hall et al., 2000), which suggests a role for canonical-Wnt signalling in presynaptic differentiation. To probe the mechanisms behind this effect and to examine the consequence of short-term blockade of canonical-Wnt signalling in hippocampal neurons, the secreted Wnt antagonist, Dkk1, was used. Young hippocampal cultures (5-7 DIV) were treated with Wnt7b conditioned media (CM), Wnt7bCM with Dkk1 (20ng/mL) or Dkk1 (20ng/mL) alone for 2 hours. Cultures were fixed with 4% PFA prior to immunohistochemical processing; endogenous VAMP2 was used to assess SV clustering. A series of Dkk1 concentrations were assayed to determine a robust minimum working concentration. With a 2-hour incubation period, a robust Dkk1 affect of VAMP2 puncta area loss was obtained with a 20 ng/ml concentration (Fig.4.1. panel A, control 0.27 μm^2 ± 0.01, Dkk1 20ng/ml 0.16 μm^2 ± 0.008, Dkk1 35ng/ml 0.19 μm^2 ± 0.01, Dkk1 50 ng/ml 0.16 μm^2 ± 0.007). As predicted, Wnt7bCM significantly increased the number of VAMP2 positive puncta per 100μm neurite length (73%, Fig.4.1 panels C and D, control 21.6 ± 2.1, Wnt7bCM 37.4 ± 3.7, p=0.0005). Addition of Dkk1 to the Wnt7bCM significantly reduced the number of SV clusters compared to Wnt7bCM treated (55%, Fig.4.1 panels C and D, Wnt7bCM 37.4 ± 3.7, Wnt7bCM and Dkk1 16.8 ± 2.4, p=0.00002), thus demonstrating that Dkk1 was able to inhibit the Wnt7b mediated SV clustering. Importantly Dkk1 alone significantly reduced the number of SV clusters

compared to control cells (59%, Fig.4.1 panels C and D, Control 21.6 ± 2.1, Dkk1 11.6 ± 1.7, p=0.0006). This data demonstrates for the first time that an endogenous canonical-Wnt activity regulates SV clustering in cultured neurons.

4.2.2.Dkk1 is not apoptotic for hippocampal neurons

The rapid loss of SV clusters, and thus putative synaptic sites, induced by Dkk1 could be interpreted in two ways. Firstly, that Dkk1 blocks Wnt7b synaptogenic properties, or secondly, that Dkk1 is neurotoxic and is inducing cell death, a process that could lead to synapse loss. Indeed, Dkk1 has been implicated in neural cell death (Busceti et al., 2007; Cappuccio et al., 2005; Caricasole et al., 2004; Mastroiacovo et al., 2009; Scali et al., 2006). To investigate whether Dkk1 induces cell death, TUNEL analysis (Terminal deoxynucleotidyltransferase-Mediated dUTP-Digoxygenin Nick End Labelling) was performed after incubating young hippocampal cultures in Dkk1 (20ng/mL). Cells were treated with Dkk1 for 2 hours, as per the incubation period in the previous experiments, and for 24 hours to ensure later stages of apoptosis would be detected. Neurons were counterstained with the nuclear marker bisBenzamide (Hoechst staining) to obtain a total cell count and the number of Hoechst positive and TUNEL positive nuclei per field of view were manually counted (Figure 4.2. panel A). The results show that at 20ng/mL, Dkk1 does not induce apoptosis in young hippocampal cultures (5-7 DIV) within a 24-hour incubation period (Figure 4.2 panel B, 2 hour control 17.9%, 2-hour Dkk1 17.8%; 24-hour control 17.1%, 24 hour Dkk1 18.2%). The average number of apoptotic cells in the control conditions is within the expected range for a dissociated hippocampal culture at this stage (Flavin et al., 1997; Meucci et al., 1998). At both 2-hours and 24-hours of Dkk1 treatment the number of TUNEL positive neurons is consistent with the vehicle (0.1% BSA) treated neurons. Therefore, Dkk1 does not induce apoptosis under these experimental conditions.

To further confirm that Dkk1 does not affect the viability of the cells, I performed "recovery" experiments. Here, young hippocampal cultures (14 DIV) were incubated with either vehicle (0.1% BSA) or Dkk1 (20ng/mL) for 2 hours; the original growing media was reserved and maintained at 37°C. Following Dkk1 or vehicle incubation, the cells were either fixed in 4% PFA or gently washed twice in warm PBS to thoroughly remove the treatment media, before further incubation in the original growing media for 48-hours. The number of VAMP2 positive puncta was quantified as previously. Analysis demonstrates a clear recovery in the number of SV clusters following Dkk1 treatment. Dkk1 induced a significant reduction of SV clusters (41% Figure 4.2 panel C, control 17.7 ± 0.7, Dkk1 13.4 ± 1.2, p=0.0046). This effect was completely ameliorated by further incubation in the original growing media (Figure 4.2 panel C, Dkk1 2 hour 13.4 ± 1.2, Dkk1+48 hour recovery 18.3 ± 1.2, p=0.0065). Importantly, the number of SV clusters following the “recovery” period is similar to the number of clusters in the equivalent control group (Figure 4.2 panel C, control+recovery 18.0 ± 1.1, Dkk1+recovery 18.3 ± 1.2). Therefore, neurons that lost 41% of their SV clusters during 2 hours of Dkk1 treatment, made a full recovery within 48 hours. Similar trends were also observed for the average area of the SV

clusters (data not shown). In summary, the ability of the neurons to regain their SV cluster population to control levels, after washout of Dkk1, corroborates the TUNEL analysis and also validates our hypothesis that Dkk1 is reducing SV clusters without affecting cell viability.

4.2.3.Inhibition of Gsk3β overrides Dkk1 activity

Inhibition of Gsk3β is a defining event in canonical-Wnt signaling (MacDonald et al., 2009; van Amerongen and Nusse, 2009; Verheyen and Gottardi, 2010). This serine/threonine kinase is downstream of Dkk1/LRP5/6 signaling at the cell surface (Figure 1.4) (Angers and Moon, 2009; Verheyen and Gottardi, 2010). To further tease out the signalling pathway, a role for Gsk3β was investigated. As work from our lab and others have shown that pharmacological inhibition of Gsk3, by lithium chloride, mimics Wnt7a/b clustering of presynaptic components (Davis et al., 2008; Hall et al., 2000), I predicated that inhibition of Gsk3 would rescue the Dkk1 effect. The rationale was that pharmacological intervention would activate the signaling pathway downstream and therefore override inhibition of the pathway at the receptor level. 6-bromoindirubin-3'-oxime (BIO) is a selective Gsk3 inhibitor that has been shown to mimic canonical-Wnt signaling (Meijer et al., 2003; Tseng et al., 2006) and with significantly greater specificity than lithium chloride (Meijer et al., 2003). For these experiments, young hippocampal cultures (14 DIV) were treated for 2-hours with vehicle (0.1% BSA or DMSO), BIO (1μg/mL) alone, Dkk1 (20ng/mL), or BIO and Dkk1 together. Presynaptic differentiation was determined by quantifying the number and area of SV clusters.

When neurons were treated with Dkk1 alone, there was a significant loss of SV clusters (32% Figure 4.3 panel A and B, control 49.0 ± 3.46, Dkk1 33.4 ± 1.93, p=0.0004). Similarly, there was a significant decrease in the average size of the SV clusters (30% Figure 4.3 panel A and C, control 0.31 μm^2 ± 0.02, Dkk1 0.21 μm^2 ± 0.01, p=0.0007). When the neurons were treated with BIO, there was no significant change to the number of SV clusters, but there was an increase in the average size of the SV clusters (20% Figure 4.3 panel A and C, control 0.31 μm^2 ± 0.02, BIO 0.37 μm^2 ± 0.02, p=0.04). As predicted, BIO in the presence of Dkk1 media did not change the number of SV clusters with respect to control cells, but interestingly did increase the average puncta area (23% Figure 4.3 panel A and C, control 0.31 μm^2 ± 0.02, BIO+Dkk1 0.38 μm^2 ± 0.02, p=0.03). Importantly, addition of BIO to the Dkk1 media significantly increased both the number and size of SV clusters by 71% and 76% respectively compared to Dkk1 treated cells. In summary, these results demonstrate that inhibition of Gsk3 by BIO prevents the loss SV clusters induced by Dkk1 and suggests that downstream activation of the canonical-Wnt pathway rescues inhibition at the level of the LRP5/6 receptor.

4.2.4.Wnt-mediated SV clustering is independent of transcription

The canonical-Wnt pathway is best characterised by transcription of β-catenin responsive genes (Cadigan, 2008; MacDonald et al., 2009). This event is mediated by inhibition of Gsk3β, which

renders cytosolic β-catenin stable and able to translocate to the nucleus where it binds to TCF/LEF transcription factors (MacDonald et al., 2009; van Amerongen and Nusse, 2009). However, there is compelling evidence from our lab for a divergent canonical-Wnt pathway, which does not require transcription. Such pathways regulate cellular processes of microtubule dynamics in developing neurons (Ciani et al., 2004) and the terminal arborisation of axons (Purro et al., 2008). Regarding synaptogenesis, Wnt7b increases SV clusters in both cerebellar mossy fibres and hippocampal neurons within 15 minutes (Ahmad-Annuar et al., 2006). This very rapid response raises the question whether transcription is involved.

To investigate the role for transcription in Wnt mediated synaptic differentiation, I tested whether Wnt7b or Dkk1 activity was affected by transcriptional blockade. For these experiments, the RNA polyermerase inhibitor Actinomycin-D (AmD) was used. To test AmD efficacy for inhibiting transcription in hippocampal neurons eGFP expression was assayed. In preliminary trials I found that eGFP expression was detectable by epifluorescence 4 hours post-transfection. For the AmD efficacy assay, young hippocampal cultures were transfected with eGFP and cultured for 4 hours to initiate expression of eGFP. AmD (10μg/mL) was then added to the cells for a further 2-hours. Visual examination by epiflourescence revealed a substantial decrease in the levels of eGFP expression levels, thereby confirming that transcription had been significantly prohibited. I then tested the effect of AmD on Wnt7b and Dkk1 activity. Here, young hippocampal cultures were treated with AmD (10μg/mL) for 30-minutes to arrest transcription, this media was then replaced with Wnt7b conditioned media containing AmD or vehicle, or media containing Dkk1 (20ng/mL) plus AmD or vehicle. The neurons were then incubated for a further 2-hours. Parallel control experiments were performed using either control CM or 0.1% BSA (see Figure 4.4 panel A for scheme of the experiment). Clustering of the synaptic proteins VAMP2 was used to determine presynaptic differentiation.

Consistent with previous experiments in young neurons, 2-hour Wnt7b treatment significantly increased the number of VAMP2 puncta per 100 μm neurite length (34% Figure 4.4 panel B, control 36.5 ± 3.4, Wnt7b 48.8 ± 3.0, p=0.009), and also the average area of the SV clusters (24% Figure 4.4 panel B, control 0.31μm^2, ± 0.008, Wnt7b 0.38μm^2, ± 0.02, p=0.002). Following the Wnt7b plus AmD treatment there was a 39% increase in puncta number (Figure 4.4 panel B, control-AmD 40.8 ± 3.2, Wnt7b-AmD 56.8 ± 4.9, p=0.008) and a 74% increase in area (Figure 4.4 panel B, control-AmD 0.23μm^2, ± 0.02, Wnt7b-AD 0.39μm^2, ± 0.02, p=4.5E-09). Thus, inhibition of transcription with AmD does not affect the ability of Wnt7a to induce presynaptic SV clustering.

Dkk1 induced a significant decrease in both the number of SV clusters (27% Figure 4.4 panel B, control 36.5 ± 3.4, Dkk1 26.7 ± 3.5, p=0.05) and their average area (46%, control 0.31μm^2, ± 0.008, Dkk1 0.17μm^2, ± 0.01, p=0.001). When transcription was blocked with AmD, there was still a significant decrease in both the number of puncta (43%, control-AmD 40.8 ± 3.2, Dkk1-AD 23.3 ± 2.9, p>0.001) and their average area (24%, control-AmD 0.23μm^2, ± 0.02, Dkk1-AD 0.17μm^2, ± 0.01, p>0.001). These data demonstrate that Wnt7b-mediated SV clustering is independent of transcription, as are the loss of SV clusters induced by Dkk1. Importantly these

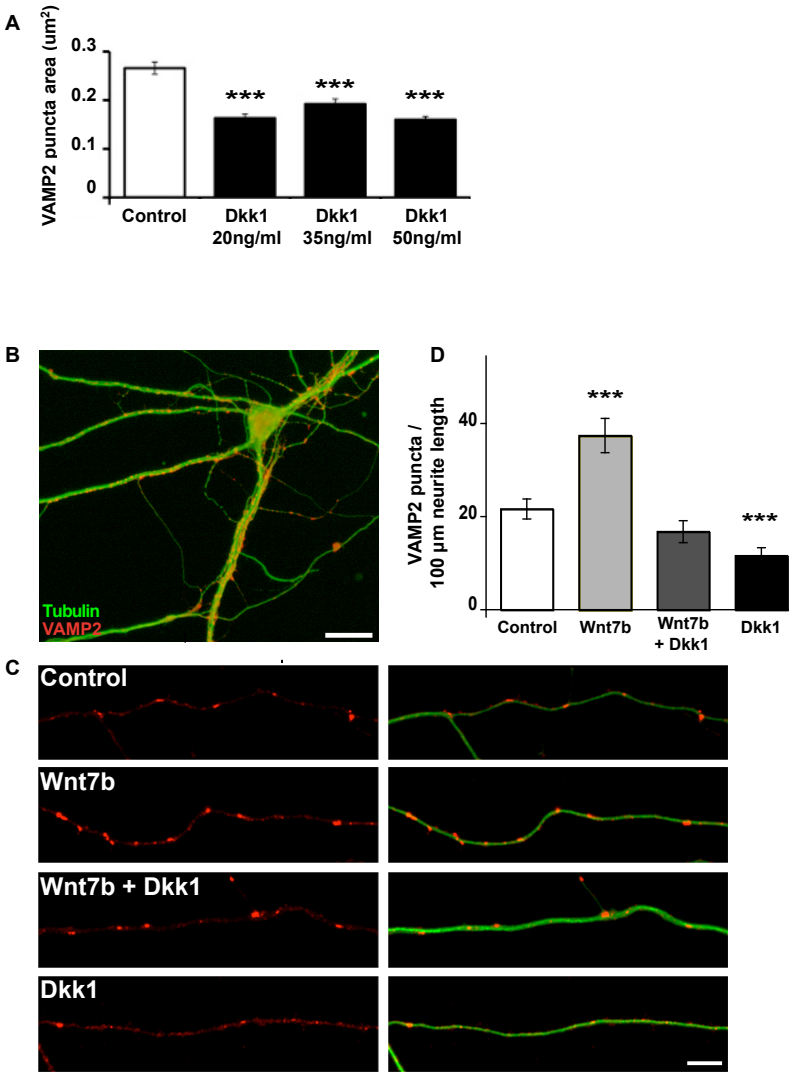
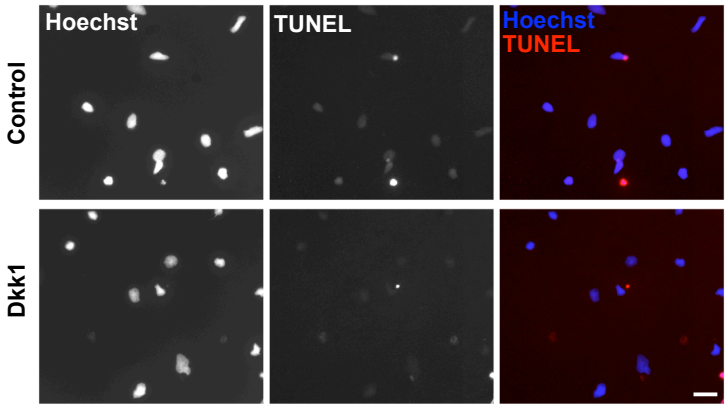


Figure 4.1: Short-Term blockade of canonical-Wnt signalling inhibits synaptic vesicle clustering. (A) Hippocampal neurons (5-7 DIV) were treated with different concentrations of Dkk1 for 2-hours to determine a working Dkk1 concentration. (B) Representative immunofluorescence from 7 DIV dissociated hippocampal culture. VAMP2 puncta labelled in red and Tubulin in green. (C) Hippocampal neurons (5-7 DIV) were treated with either vehicle, Wnt7b, Wnt7b and Dkk1 together or Dkk1 alone. Scale bars, upper panel 10 µm, lower panels 5 µm (D) Quantifications reveal that Wnt7b treated neurons have 71% more VAMP2 puncta compared to control (p=0.0005). Addition of Dkk1 to Wn7bCM inhibits the Wnt7b effect and reduces the number of VAMP2 puncta by 55% compared to Wnt7b (p=0.00002). Dkk1 alone reduces the number of VAMP2 puncta by 59% compared to control (p=0.0006). *** p<0.005 by ANOVA. Error bars represent SEM.

A



B

	Total cells	TUNEL (+)	% Apoptosis
Control	2104	377	17.9
Dkk1 2h	1691	302	17.8
Control	1724	294	17.1
Dkk1 24h	1713	312	18.2

C

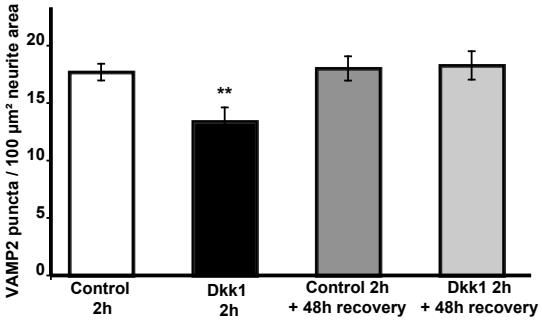


Figure 4.2: Dkk1 does not induce cell death and the effects are reversible. (A) TUNEL assays were performed on cultured hippocampal neurons after treatment with Dkk1 for 2 hours or 24 hours. Scale bar 20 µm. (B) Quantifications reveal that Dkk1 treatment does not induce apoptosis compared to control cells. (C) Hippocampal cells were treated for 2 hours with control media or Dkk1; Dkk1 was then washed out and the cells were incubated for a further 48 hours in the original growing media. Quantification of VAMP2 puncta show that although Dkk1 induced an initial decrease in SV cluster number, after 2 days in Dkk1 free media, the neurons exhibit the same number of SV clusters as the control treated neurons. Scale bar: 20 µm, **p+<0.005 by ANOVA. Error bars represent SEM.

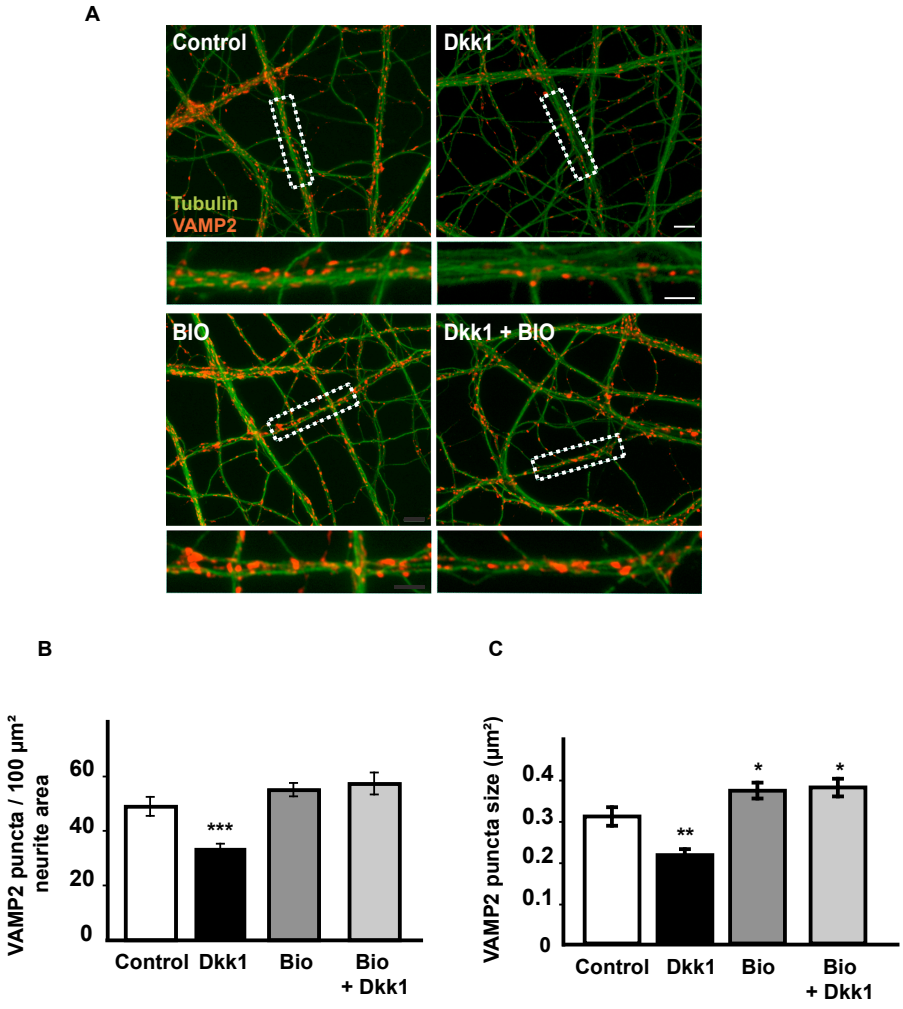


Figure 4.3: Downstream activation of the canonical-Wnt pathway blocks the Dkk1 effect on SV clusters (A) The Dkk1 effect on VAMP2 positive SV clusters (red) is inhibited by Gsk3 inhibition. Hippocampal cells were treated with Dkk1, the Gsk3 inhibitor BIO, Dkk1 and BIO, or control media. Lower panels are enlarged images from the selected areas. Scale bars: upper panels 10 µm, lower panels 5 µm. (B and C) Quantification reveals that activation of the canonical-Wnt pathway by Gsk3 inhibition blocks the ability of Dkk1 to reduce the number and size of SV clusters *p<0.05, **p<0.005, ***p<0.001 by ANOVA. Error bars represent SEM.

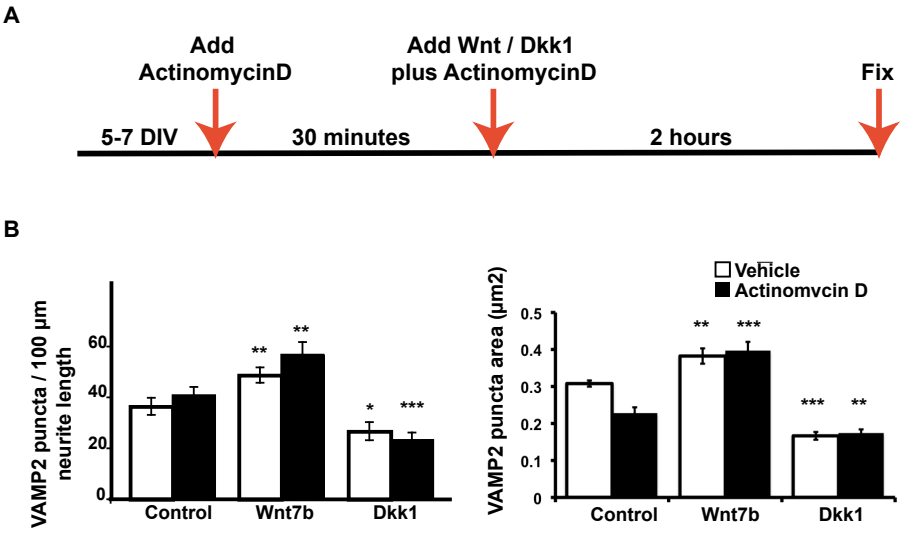


Figure 4.4: A divergent canonical-Wnt signal regulates the distribution of synaptic vesicles in hippocampal neurons. (A) Scheme outlining the experiment to block transcription with the RNA polymerase inhibitor ActinomycinD. (B) The effect of Wnt7b and Dkk1 on SV clustering is independent of transcription. In the absence or presence of ActinomycinD, Wnt7b increases the number and area of VAMP2 puncta compared to the relative control cells. Dkk1 significantly reduces the number and area of VAMP2 puncta compared to control cells in the absence or presence of ActinomycinD. Thus, the effect of Wnt7b or Dkk1 is not affected by inhibition of transcription. *p<0.05, **p<0.005, ***p<0.001 by ANOVA. Error bars represent SEM.

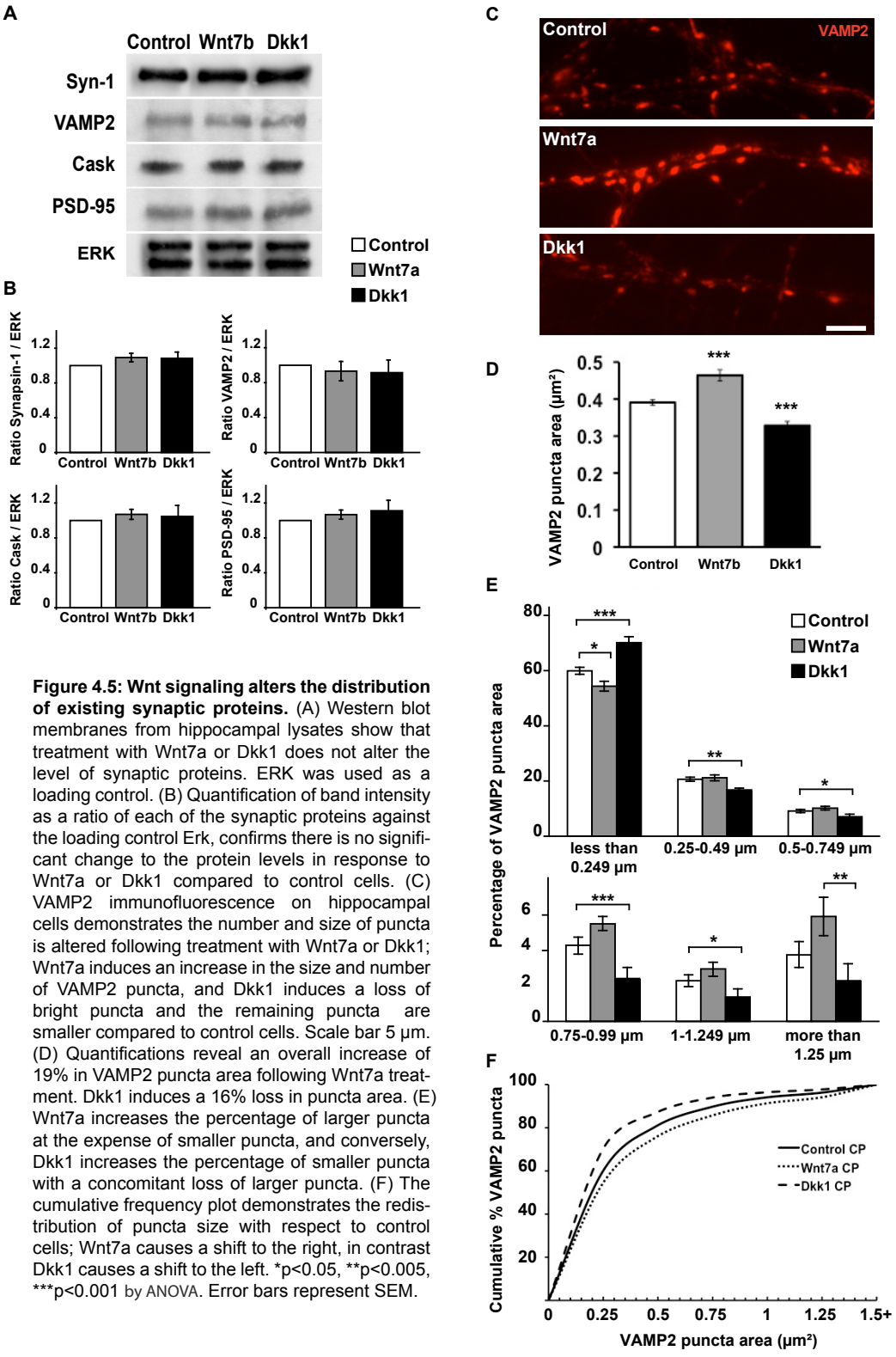


Figure 4.5: Wnt signaling alters the distribution of existing synaptic proteins. (A) Western blot membranes from hippocampal lysates show that treatment with Wnt7a or Dkk1 does not alter the level of synaptic proteins. ERK was used as a loading control. (B) Quantification of band intensity as a ratio of each of the synaptic proteins against the loading control Erk, confirms there is no significant change to the protein levels in response to Wnt7a or Dkk1 compared to control cells. (C) VAMP2 immunofluorescence on hippocampal cells demonstrates the number and size of puncta is altered following treatment with Wnt7a or Dkk1; Wnt7a induces an increase in the size and number of VAMP2 puncta, and Dkk1 induces a loss of bright puncta and the remaining puncta are smaller compared to control cells. Scale bar 5 µm. (D) Quantifications reveal an overall increase of 19% in VAMP2 puncta area following Wnt7a treatment. Dkk1 induces a 16% loss in puncta area. (E) Wnt7a increases the percentage of larger puncta at the expense of smaller puncta, and conversely, Dkk1 increases the percentage of smaller puncta with a concomitant loss of larger puncta. (F) The cumulative frequency plot demonstrates the redistribution of puncta size with respect to control cells; Wnt7a causes a shift to the right, in contrast Dkk1 causes a shift to the left. *p<0.05, **p<0.005, ***p<0.001 by ANOVA. Error bars represent SEM.

results offer the first characterization of the Wnt pathway mediating presynaptic differentiation, which is a divergent canonical-Wnt pathway.

4.2.5.Wnt signalling alters the distribution of existing synaptic proteins

Considerable evidence now supports the idea of local translation at synaptic sites, which enables and facilitates rapid synapse formation and synaptic plasticity (Shen, 2006). Therefore it is plausible that Wnt7a/b may be stimulating local translation of synaptic proteins. To examine whether changes in synaptic protein levels could explain the significant increase in VAMP2 clusters, Western blot analyses was performed. Here, several synaptic proteins were also analysed. The synaptic protein levels of neurons treated with Dkk1 were also analysed. Cell lysates were prepared from young and mature hippocampal cultures (7, 14 and 21 DIV) that had been treated with either Wnt7a (50ng/mL) or Dkk1 (20ng/mL). Following Wnt7a/Dkk1 treatment, the blots consistently revealed equivalent protein levels for VAMP2, Synapsin1, Cask and PSD95 compared to control cells (Figure 4.5 panels A and B). Erk was used as a loading control. These results suggest that Wnt7a does not stimulate significant changes in synaptic protein levels within the treatment period. Similarly, the loss of SVs induced by Dkk1 cannot be explained by significant protein loss. These data suggest that Wnt signalling may alter the distribution of existing synaptic proteins.

To further explore the notion that Wnt signalling may alter the clustering behavior of existing SVs, I investigated how addition, or blockade of Wnt affects the distribution of SV cluster size. As Wnt stimulates SV clustering in hippocampal neurons without affecting total protein levels, I predicted that addition of Wnt7a would increase the number of large SV clusters with a concomitant loss of smaller clusters (Lee et al., 2008b). Conversely, I reasoned that blockade of Wnt signaling with Dkk1 might be dispersing SV clusters; if correct this would increase the number of smaller clusters at the expense of larger clusters. Indeed, analyses reveal that Wnt induced a 9% loss of small puncta (less than 0.249 μm^2) with a concomitant 55% rise in the number of larger puncta (more than 1.25 μm^2). Conversely, neurons treated with Dkk1, revealed a 17% increase in the number of small puncta with a concomitant 40% decrease in the number of larger clusters (Figure 4.5 panels C and E). The redistribution of VAMP2 puncta size is clearly represented in the cumulative frequency plot (Figure 4.5 panel F). Wnt7a causes a shift to the right with respect to control cells indicating an increased proportion of larger puncta compared to control cells. In contrast, Dkk1 causes a shift to the left demonstrating the increased proportion of smaller puncta. These results demonstrate that Wnt signalling alters the distribution SVs, along the neurite, suggesting that Wnt regulates the recruitment of existing synaptic components to form nascent sites. Intriguingly, the results also suggest that Wnt blockade disperses larger SV clusters.

4.3. Discussion

Substantial progress has been made in the past decade in the identification of key molecules involved in synaptogenesis. However, the downstream signaling events, and therefore the underlying mechanisms, remain poorly understood. The aim of this chapter was to probe the underlying Wnt signaling pathway that mediates presynaptic differentiation. This was achieved by loss and gain of function studies using cultured hippocampal neurons. Loss of function was achieved by bath application of the secreted canonical-Wnt antagonist Dkk1. Dkk1 binds with the Wnt co-receptor LRP5/6 and blocks canonical-Wnt signalling at the receptor level (Bafico et al., 2001; Mao et al., 2001). The gain of function experiments used bath application of either Wnt7a recombinant protein or Wnt7b conditioned media (CM). The two different Wnts reflect when the experiments were actually performed; at the start of this project Wnt7a was not commercially available so CM was generated from stably transfected Rat1b cells and it's presence detected by Western blot. Wnt7a and Wnt7b stimulate presynaptic differentiaion to comparable levels (Ahmad-Annuar et al., 2006) and both are active in the canonical-Wnt pathway.

The role of Wnts as synaptogenic factors is well established. Furthermore, studies from our lab and others have shown that pharmacological inhibition of Gsk3 mimics Wnt mediated presynaptic differentiation (Ahmad-Annuar et al., 2006; Davis et al., 2008; Hall et al., 2000), which suggests a role for the canonical-Wnt signaling pathway. To further probe this issue, I performed a series of experiments to assay the role of key components in the canonical-Wnt pathway from the receptor level to the nucleus. These studies reveal a divergent canonical-Wnt pathway that is independent of transcription regulates presynaptic differentiation.

Dkk1 blocks exogenous and endogenous Wnts in hippocampal cultures. My results demonstrate that Dkk1 effectively blocks the synaptogenic activity of Wnt7b as the number of SV clusters is significantly reduced when Dkk1 is in the presence of Wnt7b. Importantly, Dkk1 alone significantly reduces the number of SV clusters compared to control cells. This suggests that endogenous canonical-Wnt proteins are required for presynaptic differentiation in cultured hippocampal neurons. Canonical-Wnt signaling is initiated by interactions between Wnt, Frizzled and the co-receptor LRP5/6. Dkk1 is a negative regulator of canonical-Wnt signaling (Glinka et al., 1998; Niehrs, 1999, 2006) as it binds specifically to LRP5/6 with great specificity and affinity (He et al., 2004) and prevents the Wnt/Fz/LRP complex from forming. Therefore, by virtue of Dkk1's mechanisms, these data purport a role for LRP5/6 in Wnt7a/b mediated synaptogenesis.

Dkk1 decreases the number of SV clusters without affecting cell viability. The results show that at working concentrations (20ng/mL) Dkk1 is not apoptotic. Apoptosis is a well-characterised mechanism that results in the death of a cell in a carefully controlled manner (Kerr et al., 1972), and is characteristic of many neurodegenerative diseases including Alzheimer's, Parkinson's, amyotrophic lateral schlerosis and Hungtington's (Vila and Przedborski, 2003). Synapse loss is

also a hallmark of these diseases (Arendt, 2009; Selkoe, 2002; Stevens et al., 2007; Yoshiyama et al., 2007) and it could be argued that the loss of SV clusters could be indicative of Dkk1 induced cell death. Indeed, a number of studies have described a potential link between cell death and Dkk1 expression induced by cerebral ischemia (Cappuccio et al., 2005; Mastroiacovo et al., 2009), epilepsy (Busceti et al., 2007) or Alzheimer's disease (Scali et al., 2006). However, my findings show that under our experimental conditions Dkk1 does not affect cell death. This conclusion is based on quantifications using the TUNEL assay. TUNEL is an immunohistochemical approach used to detect the nicked ends of fragmenting DNA, a latter stage of apoptosis. TUNEL staining is detectable within 3-hours of induced cortical ischemia with peak expression at 24-hours post insult (Ren et al., 2003); therefore TUNEL analysis following a 2-hour Dkk1 treatment may not be detected. For this reason, a 24-hour incubation period was also included in the analysis. The results clearly show that long-term Dkk1 treatment does not affect apoptosis in hippocampal neurons. Further demonstration that Dkk1 does not affect cell viability comes from recovery experiments; the loss of SV clusters was completely rescued when Dkk1 media was washed out and the neurons were allowed to recover for 48 hours. Therefore, these findings argue that Dkk1 induces SV cluster loss through a mechanism that does not involve apoptosis or changes in viability. Importantly, these data sanction Dkk1 as a tool to probe Wnt activity at the synapse.

A divergent canonical-Wnt signal regulates presynaptic differentiation. The use of Dkk1 implicates canonical-Wnt signaling, at least at the receptor level. Canonical-Wnts signal through the LRP/Fz complex to initiate a series of biochemical events (Pinson et al., 2000; Tamai et al., 2000). A key event within this cascade is inhibition of Gsk3β, which results in stable ß-catenin translocating to the nucleus, where it activates transcription (Logan and Nusse, 2004). The data presented here demonstrates that pharmacological inhibition of Gsk3, with BIO, overrides Dkk1 activity, a result that further corroborates the role of the canonical-Wnt pathway. However, SV clustering does not require transcription, as Wnt7b increases VAMP2 puncta in the presence of ActinomycinD. This finding does not repudiate a role for Wnt activated transcription for synaptogenesis, indeed transcription may be required for another Wnt mediated process at the synapse. These results focus on SV clustering, which is an early event of presynaptic differentiation (Ahmari et al., 2000; Friedman et al., 2000). The importance of this finding is that it characterizes a section of the Wnt signalling pathway that mediates synaptogenesis. The data presented in this chapter identifies a divergent-canonical pathway that signals through LRP5/6 and Gsk3β, but is independent from transcription.

The developmental stage of dissociated neuronal cultures impacts significantly on how they respond to Wnt7a/b treatments. Young cultures (5-7 DIV) show robust increases in both VAMP2 puncta size and number in response to Wnt7a/b within 2-hours. At 14 DIV, Wnt7a/b also induces an increase in VAMP2 puncta size within 2-hours, however changes in the number of detectable puncta require overnight treatment. This will in part be due to levels of endogenous secreted Wnt(s) in the culture media. Although endogenous Wnt concentrations in culture media have not in quantified, it is likely that low density immature cultures will have less

endogenous Wnt available to them compared to more developed cultures. This may make immature neurons more responsive to exogenous Wnt. In addition, immature neurons will not have the full complement of regulatory synaptogenic factors (both positive and negative) expressed. The experiments described in chapters 4.2.1 (Figure 4.1) and 4.2.4 (Figure 4.4) were performed on young cultures (5-7 DIV). The Wnt7b treatment period was 2-hours in these experiments. These data show significant increases in both puncta size and number in response to Wnt7b. In contrast, the experiments described in chapter 4.2.3 (Figure 4.3), which investigate the role of Gsk3β in synapse assembly by using the Gsk3 inhibitor BIO, were conducted on 14 DIV neurons. The BIO treatment period was 2-hours to be in line with the Dkk1 treatments. Whilst significant increases in puncta area were observed in response to BIO, no significant change in puncta number was detected. It is likely this lack of response, in terms of synapse number, is due to the age of the culture rather than an effect of Gsk3 inhibition. This discrepancy could be elucidated by overnight BIO treatment. With hindsight I should have used younger cultures in this series of experiments.

Wnt signaling alters the distribution of synaptic proteins. Western blots revealed no significant change in the total level of several pre and postsynaptic proteins in response to Wnt7a. Furthermore, the data reveals a shift in SV cluster size, which could be interpreted as smaller SV clusters coalescing to form larger neurotransmitter release sites. My data is therefore consistent with the view that Wnt7a/b induces the recruitment of synaptic proteins from an existing pool within the neurite into nascent sites. Recruitment of SVs from transit populations to nascent sites is one of the earliest events in synaptogenesis (Ahmari et al., 2000). Work from our lab has shown SV clusters can occur within 15 minutes of Wnt application (Ahmad-Annuar et al., 2006), this would suggest that Wnt signalling could be one of the early mediators of synapse assembly.

The substantial loss of SV clusters observed during Wnt blockade by Dkk1 (average 42% decrease across the experiments presented within this chapter) is not explained by any significant loss of synaptic proteins. Moreover, there is a significant shift in the spread of SV cluster size, whereby an increase in smaller SV clusters is observed at the expense of larger clusters. Importantly, this change in SV distribution is rapid and occurs within just 2 hours. It is impossible to reason that such a change is due to impedance of a synaptogenic factor. Rather, the data may represent synapse shrinkage, which could suggest blockade of endogenous Wnt disassembles existing synaptic sites. If this hypothesis were proved correct, it would don Wnt with a novel role at the synapse, one of a maintenance factor to preserve the assembly of synaptic proteins. This hypothesis is further explored in the subsequent chapters

The work presented here elucidates some of the mechanisms underpinning Wnt mediated synaptogenesis and reveals that a divergent canonical-Wnt pathway regulates early presynaptic differentiation in young cultured hippocampal neurons. This pathway has not been previously characterised and future studies will elucidate the role of Gsk3β and determine its precise mode of action. Elucidating the molecular mechanisms underlying synaptogenesis is not only

interesting in it’s own right but also presents the valuable possibility for translational research for developing therapies for neurological disorders where synaptogenesis is impeded.

CHAPTER 5

5. Short-term inhibition of endogenous Wnt signalling induces synapse disassembly in young and mature neurons

5.1. Introduction

The assembly of synaptic connections between axons and their appropriate target cells are foremost events in building functional neural circuits. However, the formation of synaptic connections in the CNS during early development is an exuberant process that generates a vast excess of synapses. Excessive or inappropriate synapses are subsequently eliminated (Goda and Davis, 2003; Lichtman and Colman, 2000). Indeed, mounting evidence now suggests that both synapse assembly and disassembly cooperate to sculpt functional neuronal circuits (Eaton and Davis, 2003; Goda and Davis, 2003; Yoshihara et al., 2009). In-vivo time-lapse recordings from the rodent neocortex have elegantly demonstrated these dynamic behaviors in the developing brain (Lendvai et al., 2000; Zuo et al., 2005a). Such studies also reveal that spine turnover decreases with age (Holtmaat 2006), whereby the majority of synapses (70-90%) in the adult brain are maintained and remain stable (Alvarez and Sabatini, 2007; Bhatt et al., 2009; Meyer et al., 2003). Whilst our knowledge of the mechanisms that control synapse assembly is rapidly expanding (McAllister, 2007; Owald and Sigrist, 2009; Waites et al., 2005), the mechanisms that regulate synapse disassembly and maintenance at the vertebrate CNS have not been studied to the same extent. Furthermore, our understanding of molecular mechanisms that directly relate the opposing forces of synapse assembly and disassembly remains poorly understood.

A synapse may be eliminated through different mechanisms; in principle these mechanisms fall into two broad categories, firstly the loss of a factor that maintains synaptic structural integrity, and secondly the expression of a factor that actively initiates disassembly. To date, much of the evidence has focused on the former alternative i.e. withdrawal of a trophic factor. Neurotrophins are key candidates for maintaining synaptic structure and numerous loss-of-function studies indicate their withdrawal results in significant synapse loss (Huang and Reichardt, 2001; McAllister et al., 1999). Specific examples include NT4 signalling via TrkB at the vertebrate NMJ (Belluardo et al., 2001; Gonzalez et al., 1999), and TGFβ signallling at the Drosophila NMJ (Aberle et al., 2002; Eaton et al., 2002). In the CNS, a number of membrane-bound synaptic organizers that are critically involved in synapse assembly continue to be expressed in the adult brain, where it is postulated they may also regulate synapse maturation and synaptic maintenance. For example, trans-synaptic complexes of neuroligin/neurexin, which adhere the pre and postsynaptic terminals and bind essential signalling and scaffold proteins (Ko et al., 2009; Varoqueaux et al., 2006); Eph/ephrin complexes, which initiate multiple complex signalling cascades and alter cytoskeletal dynamics (Klein, 2009; Lai and Ip, 2009; Lim et al.,

2008) and SynCAM, which is an adhesion molecule, that binds with multiple PDZ-domain proteins (Biederer et al., 2002). The stabilizing properties of adhesion molecules and the cytoskeleton are likely to maintain synaptic integrity by anchoring synaptic components in place.

Secreted Wnt proteins are synaptogenic factors that are essential for establishing neural circuitry (Budnik and Salinas, 2011; Ciani and Salinas, 2005; Salinas and Zou, 2008; Sanchez-Camacho and Bovolenta, 2009). A number of Wnts are strongly expressed in the developing brain, where they regulate synapse assembly by recruiting pre- and postsynaptic components to future synaptic sites (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Ciani et al., 2011; Davis et al., 2008; Farias et al., 2009; Hall et al., 2000; Henriquez et al., 2008; Krylova et al., 2002). Intriguingly, a number of Wnts are also expressed in the adult brain (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Davis et al., 2008; Farias et al., 2009; Gogolla et al., 2009; Rosso et al., 2005; Sahores et al., 2010; Shimogori et al., 2004; Varela-Nallar et al., 2009); here their role is less certain. Evidence suggests that Wnt can be released in an activity-dependent manner at both the Drosophila NMJ (Ataman et al., 2008) and the rodent hippocampus (Chen et al., 2006; Gogolla et al., 2009; Wayman et al., 2006), where it increases dendritic arborization (Wayman et al., 2006), remodels synapses (Ataman et al., 2008; Chen et al., 2006; Gogolla et al., 2009) and facilitates LTP (Chen et al., 2006). Wnt ligands initiate numerous, complex signalling cascades (Logan and Nusse, 2004; van Amerongen and Nusse, 2009). In the canonical Wnt pathway, extracellular Wnt forms a complex with its receptor Frizzled (Fz) and co-receptor LRP5/6. These interactions activate the scaffold protein Disheveled (Dvl), which subsequently inhibits the constitutive phosphorylation activity of the serine/threonine kinase Gsk3β (MacDonald et al., 2009). These events enable the transcription of target genes via β-catenin responsive transcription factors (Cadigan, 2008). Work from our lab and others have revealed a role for canonical Wnt signalling, via Gsk3β, in the regulation of presynaptic differentiation (Davis et al., 2008; Hall et al., 2002). In Chapter 4 of this thesis, my analyses revealed that Wnt increases the clustering of synaptic vesicle proteins by signaling through a divergent canonical pathway via inhibition of Gsk3β. Additionally, short-term blockade of canonical-Wnt signaling resulted in a robust loss of SV clusters without affecting protein levels. Here, I further examine this loss of putative synaptic sites in young and mature neurons. I found that inhibition of endogenous Wnt signaling by Dkk1 reduced the number of functional synaptic sites in both young and mature hippocampal neurons. Furthermore, ultrastuctural analyses revealed that the remaining synapses were significantly smaller compared to vehicle treated neurons. Combined with co-localisation analyses, these data suggest that blockade of endogenous Wnt signalling induces synapse elimination by a process of disassembly. This study uncovers a novel role for Wnt signalling in synaptic maintenance.

5.2. Short-term Wnt blockade reduces the number of pre- and postsynaptic protein clusters

Functional synapses require the coordinated activity of many different proteins assembled at the pre- and postsynaptic terminals. Therefore, to probe the evolving hypothesis that Wnt blockade by Dkk1 may induce synaptic disassembly, a range of functionally diverse pre and post synaptic markers where examined by immunofluorescence. Presynaptic markers included the SV associated proteins VAMP2 and Synapsin-1, the active zone scaffold component Bassoon and the cytomatrix scaffold protein CASK. Postsynaptically, the scaffold protein PSD-95 and the cell adhesion molecule Neuroligin2 were also assayed (Figure 5.1 panel A). In these experiments, young (14 DIV) dissociated hippocampal cultures were treated for 2-hours with the Wnt antagonist Dkk1 (20ng/mL) or vehicle (0.1% BSA) before fixation with 4% PFA and processing for immunohistochemical analyses.

Results demonstrate that the number of pre and post-synaptic puncta was significantly reduced following 2-hours of Wnt blockade by Dkk1 (Figure 5.1 panel B). Quantifications reveal these changes were highly significant (Figure 5.1. panel C); Vamp2 52% $p<0.001$, Synapsin1 65% $p<0.001$, Bassoon 50% $p<0.001$, Cask 38% $p<0.01$, PSD-95 47% $p<0.001$ and Neuroligin2 61% $p<0.001$). These results demonstrate that Dkk1 rapidly reduces the number of synaptic protein clusters on both sides of the synapse. This significant loss was observed following just 2 hours of Wnt blockade and therefore negates the interpretation that Dkk1 could only be blocking the formation of new synapses. Rather, the results are indicative of rapid synapse disassembly.

5.3. Inhibition of endogenous Wnt by Dkk1 reduces the number of functional presynaptic sites

The clustering of individual synaptic proteins is likely to represent pre- or postsynaptic sites. However, a population of these proteins may also represent clusters of synaptic proteins being transported along the neurite (Ahmari et al., 2000; Lee et al., 2008b; Sabo et al., 2006; Tao-Cheng, 2007). A defining characteristic of functional presynaptic sites is the recycling of SVs with the release of neurotransmitter (Figure 5.2 panel A). Modified styrl dyes, such as FM1-43, label plasma membrane and load SVs with fluorescent dye that can be easily visualized (Figure 5.2 panel B). This approach is used successfully to detect specific stages of SV recycling when used in conjunction with electrical or chemical stimulation to evoke SV fusion with the plasma membrane and neurotransmitter release (Betz et al., 1996). I used this dye to examine whether blockade of Wnt signalling affects the number of release sites and therefore, reduces the number of functional presynaptic sites. Young hippocampal cultures were treated for 2-hours with either Dkk1 (20ng/mL) or vehicle (0.1% BSA). Cells were then briefly depolarised with 110mM KCl in the presence of FM1-43 to evoke SV fusion with the plasma membrane and

neurotransmitter release. The cells were then fixed in 4% PFA and imaged without further processing for immunofluorescence.

The results reveal that the number of FM1-43 positive puncta per 100µm neurite length (p/nl), and therefore number of SV recycling sites, is significantly reduced during short-term Wnt blockade (59%, Figure 5.2, control 49.1 ± 5.9, Dkk1 20.0 ± 2.8, p=0.001). These data confirm that Dkk1 reduces the number of functional presynaptic terminals within 2-hours of treatment and confirm that the loss of SV clusters, as seen in the previous experiments, represent neurotransmitter release sites.

5.4. Dkk1 causes presynaptic disassembly in mature hippocampal cultures

Synapse elimination involves the progressive loss of pre- and postsynaptic components (Goda and Davis, 2003). Evidence derived from studies at the Drosophila NMJ and from rodent hippocampal cultures reveals a presynaptic program of disassembly. In both cases, one of the earliest events are the dispersal of SV associated proteins (Eaton and Davis, 2003; Eaton et al., 2002) or SVs (Hopf et al., 2002) from the synapse. To further probe the loss of synapses observed during Wnt blockade, and investigate whether a dispersal of SVs could be detected in the cultures following Dkk1 treatment, I examined the level of colocalisation between SVs and the active zone protein by immunofluorescence against VAMP2 and Bassoon respectively; these two synaptic proteins normally co-localize at synaptic sites. If Dkk1 does disperse synaptic components, I predicted that Dkk1 would delocalize SVs and the active zone. To test this prediction, mature hippocampal cultures (21 DIV) were used, as they contain a greater proportion of established, functional synapses. Cultures were treated for 2-hours with Dkk1 (20ng/mL) or vehicle (0.1% BSA) before fixation with 4% PFA and processed for immunofluorescence.

At 21 DIV, 86% of VAMP2 puncta colocalise with Bassoon and most likely represent assembled presynaptic terminals. The remaining 14% of VAMP2 puncta might represent SVs in transit (Ahmari et al., 2000; Lee et al., 2008b; Sabo et al., 2006; Tao-Cheng, 2007). Following 2-hours Dkk1 treatment there was an 86.2% increase in the level of delocalized VAMP2 puncta (Figure 5.3 panels B and C: control 17.4% ± 1.7, Dkk1 32.4% ± 1.7, p=0.0000001). These data demonstrate a significant proportion of previously co-localized SVs and active zones had segregated. Furthermore, the volume of the remaining co-localized puncta was significantly reduced (Figure 5.3 panels B, D and E). Following Dkk1 treatment there were 47% less co-localised puncta (data not shown), importantly, the remaining co-localized VAMP2 punta were 26% smaller (Figure 5.3, panel D: control 0.60 μm^3 ± 0.04, Dkk1 0.44 μm^3 ± 0.02, p=0.003) and the remaining Bassoon puncta that were colocalised were 58% smaller (Figure 5.3, panel E, control 0.73 μm^3 ± 0.09, Dkk1 0.31 μm^3 ± 0.02, p=0.00007). Together, these results suggest that a significant proportion of assembled presynaptic terminals had disassembled during short-term Wnt blockade. Furthermore, the remaining assembled synapses were significantly smaller

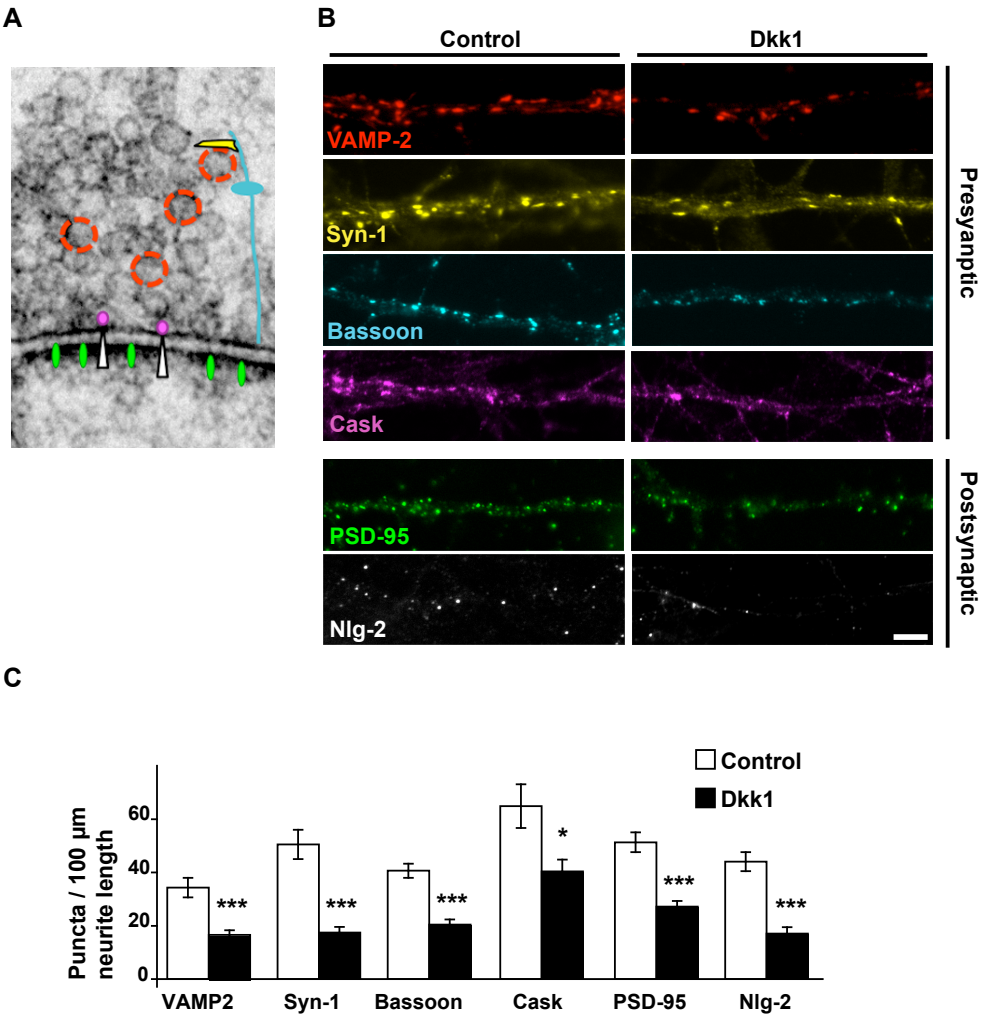


Figure 5.1: Short-term Wnt blockade reduces the number of pre- and postsynaptic protein marker clusters in cultured hippocampal neurons. (A) Electronmicrograph of a synapse to illustrate the spatial distribution of the synaptic markers assayed. NB this is not to scale, VAMP2 (red), Synapsin1 (yellow), Bassoon (cyan), Cask (pink), PSD95 (green), Neuroligin2 (white). (B) Immunofluoresence against different pre and postsynaptic protein markers demonstrated a loss of cluster sites at 14 DIV following 2-hours Dkk1 treatment. (C) Quantification of puncta per 100 µm neurite length for each of the synaptic markers revealed significant reductions for all of the markers *p<0.05, *** p<0.001 by ANOVA. Scale bar 5 µm. Error bars represent SEM.

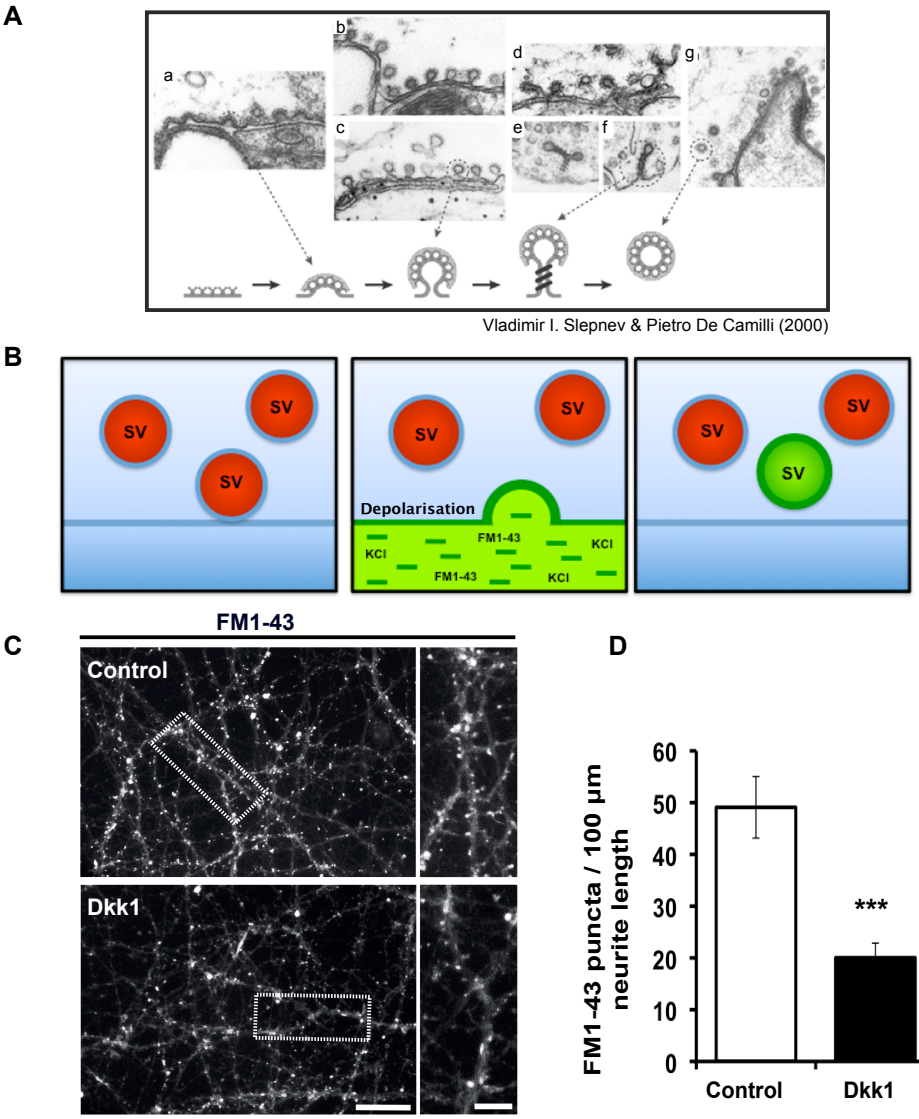


Figure 5.2: Inhibition of endogenous Wnt reduces the number of functional presynaptic sites. (A) Stages of clatherin-mediated endocytosis at the presynaptic terminal (a-g) Electron micrographs of Lamprey reticulospinal synapses microinjected with antibodies against accessory proteins required for clatherin-mediated endocytosis. (a-d) Early stages of SV retrieval from the plasma membrane. (e-f) SVs bud off from the membrane. (g) Retrieved SVs following endocytosis (B) Cartoon illustrates the FM1-43 experiment. Left, SVs close to the plasma membrane in the presynaptic terminal. Middle, cells chemically stimulated with KCl in the presence of FM1-43 evoke SV fusion with the plasma membrane. FM1-43 binds to the outer layer of the plasma membrane and the exposed luminal membrane of SVs. Right, The FM1-43 labeled SV is retrieved by endocytosis and the cells are washed to remove excess dye from the cleft and exposed cellular membranes. The labeled SVs can then be visualized by fluorescent microscopy. (C) FM1-43 uptake demonstrated that Dkk1 treatment reduced the number of SV recycling sites (puncta). Right panels are enlarged images from the selected areas. Scale bars: left panels, 20 µm, right panels, 10 µm. (D) Quantifications reveal a 59% decrease in the number of SV recycling sites within 2-hours of Dkk1 treatment ($p<0.001$ by ANOVA). Error bars represent SEM.

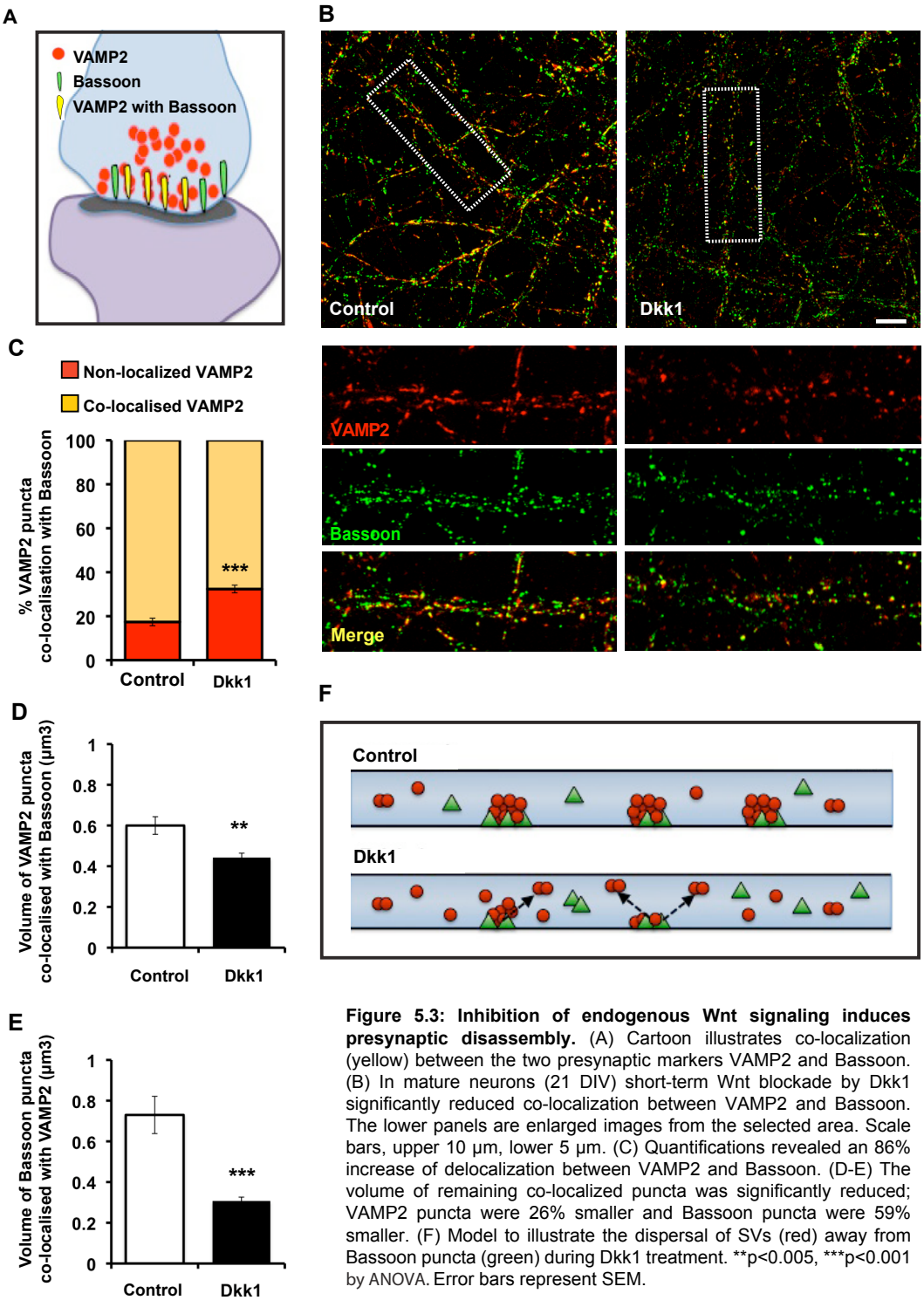


Figure 5.3: Inhibition of endogenous Wnt signaling induces presynaptic disassembly. (A) Cartoon illustrates co-localization (yellow) between the two presynaptic markers VAMP2 and Bassoon. (B) In mature neurons (21 DIV) short-term Wnt blockade by Dkk1 significantly reduced co-localization between VAMP2 and Bassoon. The lower panels are enlarged images from the selected area. Scale bars, upper 10 µm, lower 5 µm. (C) Quantifications revealed an 86% increase of delocalization between VAMP2 and Bassoon. (D-E) The volume of remaining co-localized puncta was significantly reduced; VAMP2 puncta were 26% smaller and Bassoon puncta were 59% smaller. (F) Model to illustrate the dispersal of SVs (red) away from Bassoon puncta (green) during Dkk1 treatment. **p<0.005, ***p<0.001 by ANOVA. Error bars represent SEM.

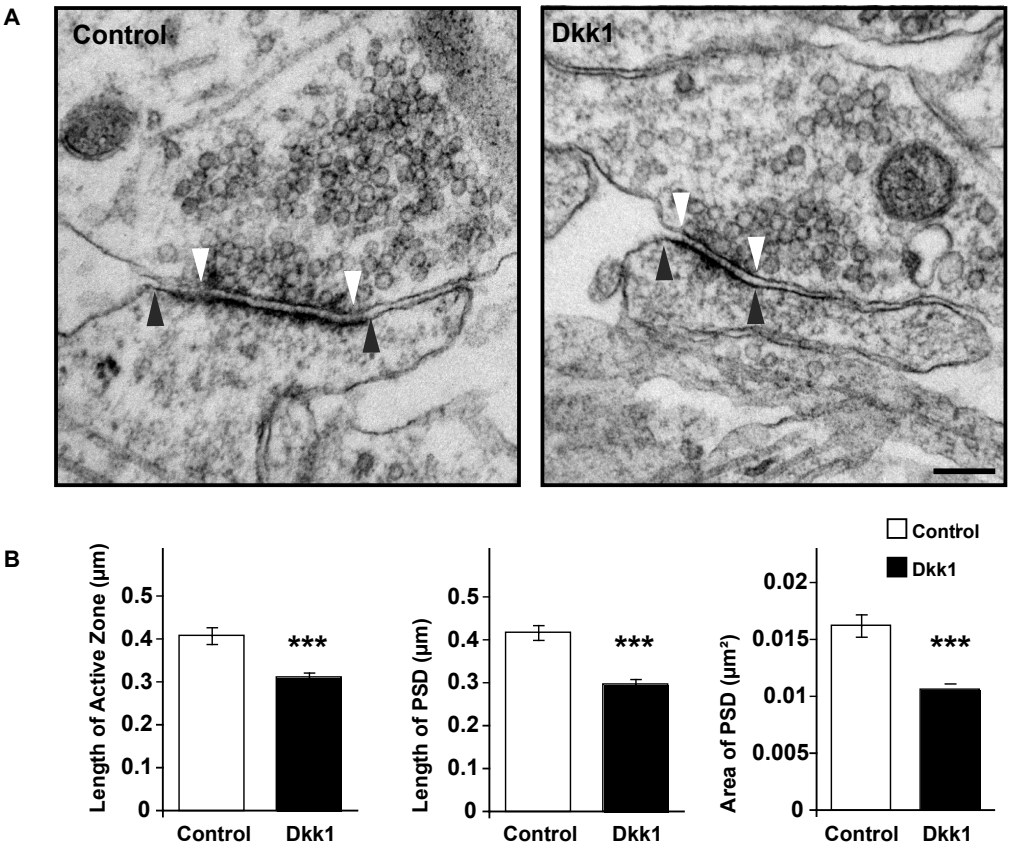


Figure 5.4: Dkk1 rapidly reduces the size of synapses in mature hippocampal cultures. (A) Electronmicrographs of mature cultured hippocampal neurons (21 DIV) treated with vehicle or Dkk1. White arrowheads indicate the length of the active zone (AZ) and black arrowheads indicate the length of the postsynaptic density region (PSD). Scale bar, 0.2 µm. (B) Quantifications revealed a significant reduction in size of the AZ and PSD within 2-hours of Wnt blockade; length of AZ reduced by 24%, length of PSD by 29%, area of PSD by 35%. (C) Electronmicrographs of asymmetric (upper panel) and symmetric (lower panel) synapses. Only asymmetric synapses were included in the quantifications. ***p<0.001 by ANOVA. Error bars represent SEM.

suggesting a progressive loss of SVs and active zone protein from synaptic sites (Figure 5.3 panel F).

5.5. Inhibition of Wnt signalling causes synapse shrinkage

To further investigate the hypothesis that inhibition of Wnt-signaling disassembles bona fide synaptic sites, and to gain further insight into what may be happening ultrastructurally, mature cultured hippocampal neurons were analyzed by electromicroscopy. The classical criterion for defining a synapse at the ultrastructural level dictates the following structures must be observed; a pool of synaptic vesicles associated with plasma membrane that is directly opposed to a PSD and separated by a defined synaptic cleft (Colonnier, 1968; Gray, 1959; Harris et al., 1992). Therefore, with these stringent classification criteria synapses in the advanced stages disassembly would be excluded. Asymmetrical synapses (Gray, 1959) were selected for analyses to enable PSD quantifications (Figure 5.5 panel C). In addition to the PSD area, measurements of the PSD length and AZ length were also made. In these experiments, mature (21 DIV) hippocampal cultures were treated for 2-hours with Dkk1 (20ng/mL) or vehicle (0.1% BSA) before fixation. I took the samples through the complete fixation protocol until they were mounted on to the resin block. A senior electron microscope technician within the Department further processed the samples. I acquired all the images blind and remained blind until the analyses were completed. Although neurons were initially plated as a monolayer, processes do grow over and under one another and create uneven depths of tissue. I therefore used the most basal sections for analyses to ensure continuity. Coverslips were always placed centrally over the resin block prior to cutting to ensure similar regions of the coverslip were consistently analysed.

Quantifications from the electronmicrographs reveal the length of AZ was significantly reduced (23%, Figure 5.5, control 0.41μm ± 0.017 n=141, Dkk1 0.31μm ± 0.011 n=103, $p<0.001$). In addition, the length of the PSD was reduced (29%, Figure 5.5, control 0.42μm ± 0.017 n=152, Dkk1 0.30μm ± 0.012 n=144, $p<0.001$). Importantly, the area of the PSD was also significantly reduced (35%, Figure 5.5, control 0.02μm^2 ± 0.0001 n=152, Dkk1 0.01μm^2 ± 0.0006 n=144, $p<0.001$). These results demonstrate that the remaining synapses, following Dkk1 treatment, were significantly smaller compared to control cells. Importantly, both pre- and postsynaptic components had shrunk during the 2-hour treatment period. Taken together with the immunofluorescence above, these data suggest that synapse shrinkage may precede elimination, as a result of short-term Wnt blockade.

5.6. Discussion

Significant progress has been made in elucidating the molecules and mechanisms involved in synapse assembly. However, synapses are remarkably dynamic structures; they assemble, remodel and disassemble both in early development and throughout life (Eaton and Davis,

2003; Holtmaat et al., 2005; Trachtenberg et al., 2002). Synaptic plasticity is believed to involve the strengthening and weakening of existing synapses, which involves enlargement and shrinkage of synapses (Bastrikova et al., 2008; Segal, 2005; Steiner et al., 2008; Zhou et al., 2004) and structural plasticity is achieved by the formation and elimination of synapses (Holtmaat and Svoboda, 2009). Intriguingly, it has been suggested that synapse modification may be a cellular substrate for learning and memory (Bailey and Kandel, 1993; Lichtman and Colman, 2000). Whilst considerable progress is being made in understanding the mechanisms of synapse assembly and structural modifications, the molecular cues and mechanisms that regulate synaptic maintenance and disassembly remain poorly understood. Here I present data that strongly suggests short-term blockade of endogenous Wnt signalling induces synapse elimination, by a process of protein disassembly, in young and mature hippocampal neurons.

Secreted Wnt proteins are synaptogenic factors that stimulate the recruitment of pre- and postsynaptic proteins in the developing vertebrate brain (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Ciani et al., 2011; Davis et al., 2008; Farias et al., 2009; Hall et al., 2000). Furthermore, evidence suggests that Wnt signalling regulates synapse assembly and remodeling in the adult brain (Gogolla et al., 2009). In this study, I show that inhibition of endogenous Wnt, by the secreted Wnt antagonist Dkk1, decreases the number synaptic sites as revealed by the significant loss of many functionally diverse pre- and postsynaptic protein clusters. The analyses reveal a loss of SVs, a loss of proteins that tether SVs to the cytomatrix, a loss of active zone scaffold protein and, importantly, the loss of a major postsynaptic scaffold protein and a CAM. Importantly, this loss of synaptic markers was observed within 2-hours of Wnt inhibition. This rapid effect counteracts the argument that Dkk1 is only blocking the formation of new synaptic sites. Indeed, it was this rapid loss of synaptic markers that led to the hypothesis of Wnt mediated synaptic maintenance.

Short-term blockade of endogenous Wnt induces the elimination of neurotransmitter release sites and the dispersal of presynaptic components. FM1-43 fluorescence imaging methods are widely used to study the function of isolated boutons with high throughput (Friedman et al., 2000; Hopf et al., 2002; Murthy et al., 1997; Ryan et al., 1993; Ryan and Smith, 1995). The significant loss of FM1-43 puncta following Wnt blockade clearly demonstrates a loss of neurotransmitter release sites indicating the disassembly of functional presynaptic sites. The notion that Dkk1 induces dispersal of SVs from synaptic sites was explored by examining the co-localization of SVs with the active zone scaffold protein Bassoon. Within 2-hours of Wnt blockade, there was a significant increase in the number of SV clusters that did not localize with the active zone. This implies that a significant proportion of the SV clusters were no longer associated with synaptic sites and suggests presynaptic disassembly had occurred or was in progress. Bassoon is documented as a stable component of the presynaptic terminal (Tao-Cheng, 2006a; Tsuriel et al., 2009). In contrast, SV pools are highly dynamic (Darcy et al., 2006; Fernandez-Alfonso and Ryan, 2008; Hopf et al., 2002; Westphal et al., 2008). Therefore, whilst this co-localization analysis cannot determine a hierarchy of disassembly, it is likely that Dkk1 induced SV dispersal away from the active zone. Size analysis of the co-localized puncta

revealed a prodigious reduction of SV pool size and Bassoon cluster volume. This suggests that the remaining assembled presynaptic sites had shrunk considerably in size. Taken together, the data suggests that progressive synapse disassembly may precede synapse elimination during Wnt blockade.

Synapses shrink during Wnt blockade. Analyses at the ultrastructural level is consistent with the above finding that synapses that were not eliminated had significantly shrunk in size; the active zone was significantly smaller and there was considerable loss of PSD material. To prudently determine synaptic changes at the ultrastructural level, it was necessary to adopt the classical criteria to identify bona fide synapses, that is; observation of homogenous SV pool associated with an active zone apposed to a PSD. The active zone contains the complex proteomic machinery for SV fusion and neurotransmitter release (Landis et al., 1988; Siksou et al., 2007). These structures must be opposed to the electron dense PSD, but be physically separated by a visible cleft (~20nm). The PSD is an electron dense region that contains neurotransmitter receptors, ion channels, scaffold proteins and a plethora of associated proteins (Sheng and Hoogenraad, 2007). Synapses are ultrastructurally classified into two broad categories based on the PSD; symmetrical and asymmetrical (Colonnier, 1968; Gray, 1959), which are understood to represent excitatory and inhibitory synapses respectively. Excitatory synapses have a thicker, well-defined PSD giving an asymmetrical appearance, and inhibitory synapses have a considerably thinner PSD (Gray, 1959) (see Figure 5.5 for representative examples). Therefore, to characterize any postsynaptic effects, during Wnt blockade, only readily identifiable, asymmetrical synapses were selected for analysis. With these stringent classification criteria in place, there are significant limitations that should be noted; firstly, only effects on excitatory synapses may be extrapolated. Secondly, synapses in the advanced stages of disassembly may no longer fit the selection criteria, for example the SV pool may have dispersed or the AZ or PSD may have dismantled to an extent whereby its presence is questionable. Therefore it is important to note that the quantifications only describe the remaining, assembled synapses. Surprisingly, with all of the imposed restrictions, quantifications still revealed a significant shrinkage of both pre and postsynaptic components during short-term Wnt blockade. In accordance with existing literature, the AZ and PSD were accurately size matched (Harris et al., 1992; Schikorski and Stevens, 1997), and interestingly following Wnt blockade, the AZ and PSD remained size matched, albeit a smaller size match. This suggests both the pre- and postsynaptic components reduced in size at comparable rates and that Dkk1 induced synapse disassembly is a co-coordinated event. Indeed, studies have shown that synapse shrinkage associated with LTD affects the size of spines (Nagerl et al., 2004; Zhou et al., 2004) and boutons (Becker et al., 2008). Future work will determine whether Wnt signaling is required at both the pre and postsynaptic compartments to maintain the SV pool, the AZ and PSD, or whether blockade at one of the terminals is sufficient to induce pre- and postsynaptic disassembly and elimination.

Synapse elimination involves a progressive loss of pre- and postsynaptic components from synaptic sites (Goda and Davis, 2003). However, it has been argued that passive disassembly,

resulting from withdrawal of a maintenance factor, could not account for the rapid loss of all synapses (Eaton and Davis, 2003; Goda and Davis, 2003). Disruption of the dynamic cytoskeleton would permit rapid synapse disassembly. Actin is enriched both pre- and postsynaptically and evidence suggests it is a critical player in modulating synapse stability (Luo, 2002; Matus, 2000; Tanaka et al., 2000) and function (Cingolani and Goda, 2008; Morales et al., 2000). In addition microtubules may well participate in stabilizing the synapse (Eaton et al., 2002). Intriguingly, Wnt signalling has been shown to modulate cell behaviors such as cell adhesion, morphology, migration and structural remodeling, by regulating cytoskeletal dynamics (Akiyama and Kawasaki, 2006; Ciani et al., 2004; Purro et al., 2008). It is tempting to speculate that this may be a mechanism for Wnt mediated synapse disassembly. Could inhibition of Wnts alter the cytoskeletal architecture and jeopardize synapse stability? A cytoskeletal web, comprising actin and other structural components, may cluster the reserve pool of SVs close to the synaptic junction (Landis et al., 1988; Phillips et al., 2001; Siksou et al., 2007), yet conclusive studies have yet to demonstrate the effects of disturbing the native dynamics of this network at the synapse during disassembly.

Could Dkk1 be disassembling synapses by mechanisms other than Wnt inhibition? Whilst there is a compelling body of work that characterizes Dkk1 as robust and potent Wnt antagonist via LRP5/6 interactions (Niehrs, 2006), it cannot be ruled out that Dkk1 could be inducing synapse disassembly by alternative signaling pathways. Dkk1 has been shown to stimulate JNK signaling via the PCP pathway in cardiogenic explant tissue and Ewing tumor cells (Endo et al., 2008; Pandur et al., 2002). However, the mechanism for Dkk1 signaling in these cases is via LRP5/6 binding to block canonical-Wnt pathway. Then, provided the necessary signaling intermediates are present, Wnts can signal via the PCP pathway to activate JNK (Zorn, 2001). Other studies have shown that Dkk1 can function independently of β-catenin, suggesting a role outside of canonical-Wnt signaling. For example, Dkk1 induces apoptosis and suppresses growth in mesothelioma H28 cell lines, which contain a β-catenin deletion, (Lee et al., 2004). Furthermore, in HeLa cells Dkk1 arrests cell transformation without affecting β-catenin localization or expression of Wnt target genes (Mikheev et al., 2004). Whilst these studies suggest Dkk1 can function independently of traditional canonical-Wnt blockade, it can be argued that Dkk1 is blocking divergent canonical-Wnt pathways that are independent of β-catenin. This thesis work, and other research studies already discussed, describe a divergent canonical-Wnt signaling pathway. Indeed this thesis is the first description of Dkk1 blocking a pathway other than canonical-Wnt signaling. Can Dkk1 signal independently of LRP5/6? To date there is no evidence for this, and binding sites for receptors other than LRP5/6 and Kremen have not been characterized. However, further research is required to fully examine this possibility. I have shown that Dkk1 blocks Wnt7a/b induced presynaptic differentiation and induces synapse shrinkage and disassembly by a process that does not involve apoptosis. Furthermore, I have shown that inhibition of Gsk3 rescues Dkk1 induced synapse loss. Whilst it cannot be ruled out that Dkk1 may signal independently of Wnt antagonism to affect synapse stability, there is no convincing evidence published that this could be the case.

In conclusion, the data presented in this study demonstrate that inhibition of endogenous Wnt signalling eliminates synapses within 2-hours; the remaining synapses are significantly smaller, both pre and postsynaptically, which suggests a process of progressive disassembly. Extensive synapse loss is a hallmark of neurodegenerative disorders such as Alzheimer's, senile dementia and Parkinson's disease (Day et al., 2006; Selkoe, 2002). Furthermore, it has recently been postulated that synapse loss may actually precede neuronal death and could be a cause of neuronal death rather than its consequence (Selkoe, 2002; Stevens et al., 2007; Yoshiyama et al., 2007). This could suggest the initial pathology of such devastating disorders lies within synaptic function and stability. Therefore, understanding the mechanisms that determine whether nascent synapses are stabilized or eliminated, or whether established synapses are maintained or disassembled will provide important insights into the initial phases of neurodegenerative disorders and also how the healthy brain reorganizes its circuitry throughout life.

CHAPTER 6:

6. Wnt-blockade de-stabilizes presynaptic vesicle clusters in mature hippocampal neurons

6.1. Introduction

The stabilization or elimination of synapses are critical processes that determine circuit refinement and function in the developing and mature peripheral and central nervous systems (Chen and Regehr, 2000; Constantine-Paton et al., 1990; Fraser, 1992; Katz and Shatz, 1996; Lichtman, 1977; Purves and Lichtman, 1980). The powerful combination of fluorescent-tagged proteins and confocal time-lapse microscopy has heralded the way forward in the study of synapse dynamics. Long-term, high-resolution imaging studies *in-vitro* and *in-vivo* have demonstrated that the majority of dendritic spines and axonal boutons in the adult brain are relatively stable, yet a sizeable population continues to rapidly assemble and disassemble. Most of these new events are transient but studies focused on spine dynamics have revealed newly formed spines stabilize in response to sensory experience (Holtmaat and Svoboda, 2009), and new persistent spines partner with presynaptic boutons to form stable and functional synaptic sites (Knott et al., 2006; Nagerl et al., 2007). What determines whether established and nascent synaptic contacts are stabilized or eliminated? To date, the molecular mechanisms that regulate this critical process remain poorly understood.

A fundamental role for presynaptic terminals and boutons is neurotransmitter release. This function is achieved via SV fusion with the plasma membrane, whereby neurotransmitter molecules, contained within SVs, are released into the synaptic cleft. Presynaptic SV clusters are highly dynamic (Bamji et al., 2006; Kalla et al., 2006; Krueger et al., 2003; Tsuriel et al., 2006). Characterization of spatiotemporal SV trafficking and mobilization reveal lateral movement of SVs in an activity dependent manner (Becker et al., 2008; Frischknecht et al., 2008; Li and Murthy, 2001; Staras et al., 2010) and SVs may even be shared between neighboring synapses (Becker et al., 2008; Darcy et al., 2006; Fernandez-Alfonso and Ryan, 2008; Lee et al., 2008b; Staras et al., 2010). However, for synaptic function to be maintained a pool of SVs must persist at the active zone, therefore mechanisms exist to recruit and preserve SV populations at synaptic sites. SV dynamics and stability are likely to depend upon the integrated activity of many functionally diverse factors, including interactions between secreted proteins, homo- and heterophyllic cell adhesion molecules, scaffold proteins and effectors of cytoskeletal dynamics (Eaton et al., 2002; Piechotta et al., 2006; Renner et al., 2008; Schuster et al., 1996; Tanaka et al., 2000). However, the mechanisms that regulate SV mobility, recruitment and retention at the synapse have yet to be fully elucidated.

Data presented in Chapters 4 and 5 of this thesis suggest that blockade of Wnt signaling by Dkk1 disassembles established synaptic sites within 2-hours. My results show that Dkk1 induced a loss of larger SV clusters with a concomitant gain of smaller SV clusters, suggesting SVs disperse from larger cluster sites during Wnt blockade. SV dispersal was associated with the elimination of neurotransmitter release sites, active zone material and pre- and postsynaptic scaffolding proteins. Furthermore, remaining synapses showed significant shrinkage of the active zone and PSD. The rapid loss of synaptic proteins occurred without detectable losses in total protein level suggesting that Dkk1 induces synapse disassembly, whereby synaptic proteins disperse from synaptic sites as opposed to being degraded. These data suggest that endogenous Wnt signaling plays a role in regulating the stability of SV clusters and synaptic sites.

To further examine a role for Wnt mediated synapse stability, and observe SV dynamics and dispersal during Wnt blockade by Dkk1, I imaged presynaptic SV clusters in mature hippocampal neurons (21 DIV) expressing VAMP2-mRFP with time-lapse confocal microscopy. SV cluster dynamics have been imaged in a number of studies to visualize synapse assembly and disassembly in real time (Ahmari et al., 2000; Alsina et al., 2001; Gomes et al., 2006; Hu et al., 2005; Nonet, 1999; Sabo et al., 2006). Here I present evidence that Wnt-blockade rapidly alters basal SV cluster dynamics in mature cultured hippocampal neurons. In control conditions SV cluster size fluctuated considerably, but there was no net increase or decrease in size by the end of the 1-hour recording period and all of the clusters imaged persisted throughout the recording period. In contrast, disruption of Wnt signaling reduced the size of stable SV clusters within 10-20 minutes and induced complete cluster elimination of almost half of the stable clusters observed within 1 hour. SV clusters failed to sustain any increases in size during Wnt blockade, and cluster sizes progressively decreased. Wnt blockade affected ~90% of stable SV clusters and affected SV clusters of all sizes. These data corroborate the results presented in Chapter 5 that Wnt blockade induces synapse disassembly and supports the hypothesis that endogenous Wnt signaling regulates synapse stability in mature hippocampal neurons. Importantly, the data in this chapter reveals a rapid timecourse of SV cluster disassembly in response to Wnt blockade, which suggests endogenous Wnt signaling regulates synapse stability through local mechanisms. A role for Wnt signaling in synaptic maintenance at mature synapses is a novel finding for Wnt activity in the CNS.

6.2. Results

Time-lapse imaging demonstrates that SV clusters rapidly fluctuate in size (Becker et al., 2008; Hopf et al., 2002; Lee et al., 2008b; Staras et al., 2010). However, the mechanisms that regulate such behavior are poorly understood, and the mechanisms that ensure stable clusters of SVs remain at synaptic sites for neurotransmitter release have yet to be fully elucidated. SV dispersal from stable cluster sites is described as an early event in presynaptic disassembly (Hopf et al., 2002). If Wnt blockade induces synapse disassembly, I predicted that time-lapse

microscopy would capture the dispersal of SVs from stable SV clusters and reveal an accurate timecourse for Wnt mediated synapse disassembly. Dissociated hippocampal neurons were transfected with VAMP2-mRFP to label SV clusters and cultured for 21-23 DIV. Mature neuronal cultures were used in these experiments to increase the probability of observing established, functional synapses. For confocal time-lapse microscopy, stacks of 8-10 images were acquired at 5-10 minute intervals. The first 20 minutes prior to addition of control or Dkk1 media (pre-recording) was used to identify "stable" SV clusters. These clusters were selected for observation and analysis, as they were most likely to represent functional presynaptic sites.

Note for the Supplemental Data: In both the control (SV1) and Dkk1 (SV2) time-lapse movies, there is a blank frame shortly after the movie begins. This is where the time-lapse was paused to exchange the media.

6.2.1. Blockade of Wnt signaling alters the dynamic behavior of SV clusters

On average 41.1% (± 5.6) of the total SV clusters in the field of view were found to be stable (data not shown). The remaining puncta were remarkably mobile and could be seen to move along neurites in real time, these puncta were classified as "in transit" and were not selected for analysis (see Supplemental movie). Stable SV clusters were categorized as either no change in area (±10%), increased in area (>10%), decreased in area (>10%) or disassembled. Results from the time-lapse imaging demonstrate the dynamic behaviors of stable SV pools (Figure 6.1). In a preliminary trial experiment where frames were acquired every minute, the fine detail of how SV cluster size fluctuates over time can be observed (Figure 6.1 panel A). This dynamic behavior is reflected in the kymographs (Figure 6.1 panel B). In future experiments, frames where acquired very 5-10 minutes to reduce the chance of photobleaching the VAMP2-RFP. Importantly, the kymographs demonstrate that photobleaching did not occur as SV clusters that were lost during the recording were next to clusters that remained fluorescently intense. Therefore the loss of SV clusters cannot be argued as experimental artifacts.

Quantifications show that in control neurons, the percent of stable puncta that did not change in area (±10%) remained moderately constant throughout the 60-minute recording period (Figure 6.1 panel C). These quantifications are relative to the initial cluster area measured at the start of the pre-recording period. Similarly, the population of puncta that decreased in area remained reasonably constant. Whilst there was a slight increase in the population of puncta that grew in area over the recording period this change was not statistically significant. The percent of puncta that disassembled during the experiments remained absolutely constant at 0%.

In contrast, during Wnt blockade with Dkk1 there was a steady decline in the puncta population that did not change in size. Similarly there was a decline in the puncta population that increased in size. This finding suggests that SV clusters are unable to grow or maintain their size in the absence of endogenous Wnts. The steady decline of SV clusters that remained constant or increased in size was coupled with a steady incline in the puncta population that became

smaller in size. Furthermore, the population of SV clusters that disassembled during the 60-minute recording period steadily increased from 0% to 49% of the population (Figure 6.1 panel C, also see Supplemental movie). Therefore during Wnt blockade, progressively more SV clusters became smaller and disassembled at the expense of clusters that grow or remain constant in size. Importantly, the changes observed in the dynamic behavior of SV clusters during Wnt blockade occurred as soon as the media was exchanged. Therefore these data reveal that blockade of endogenous Wnt signaling by Dkk1 rapidly modifies the dynamic behavior of SV clusters and induces the disassembly of previously stable clusters.

6.2.2.Wnt blockade induces SV dispersal from stable cluster sites

To clearly visualize the level of VAMP2-mRFP within stable SV clusters, the image frames were converted to pseudo color. Changes of intensity at individual puncta can be interpreted as the relative number of SVs present within the cluster. In control neurons, the intensity of VAMP2-mRFP puncta fluctuated slightly but there was no significant change by the end of the recording period (Figure 6.2 panel A). This relative stability is reflected in the quantification of puncta area (Figure 6.2 panel B). In contrast, in the presence of Dkk1, a loss of VAMP2-RFP intensity was observed at many puncta within 10 minutes (Figure 6.2 panel A). Quantifications revealed a steady loss SV cluster area after Dkk1 addition, with a rapid shrinkage of cluster size after 20-minutes. I found an overall decrease of SV cluster area of 56% ±13.5% by the end of the 60-minute recording period (Figure 6.2 panel B). These findings demonstrate that SVs rapidly disperse from stable cluster sites during Wnt blockade resulting in progressive shrinkage of SV cluster size.

A graph depicting the change in SV cluster area for representative stable puncta during control and Dkk1 conditions illustrates the behavior of individual SV clusters. In control cells, the larger cluster (~0.6 μm^2) maintained a constant size over the 20-minute pre-recording and 60-minute recording period, whereas the smaller cluster grew in size. In contrast, during Wnt blockade both the large and small cluster decreased in size; the large cluster was completely disassembled within 15-minutes of exposure to Dkk1, and the small cluster was disassembled within 60-minutes. Scrutiny of individual puncta reveals how SV cluster size rapidly fluctuates over 5-minute intervals and underscores the dynamic behavior of SVs.

6.2.3.Wnt blockade induces rapid elimination of stable SV clusters

The results described above reveal that blockade of endogenous Wnt signaling by Dkk1 causes stable SV clusters to shrink in size. To further explore the disassembly and elimination of SV clusters over time, analysis on the number of stable puncta was performed. Results show a significant loss of stable SV clusters 20 to 30 minutes after Dkk1 addition (Figure 6.3 panel A and B), followed by the progressive elimination of clusters until the end of the 60-minute recording period (Figure 6.3 panel B). I next examined whether Dkk1 differentially affects SV

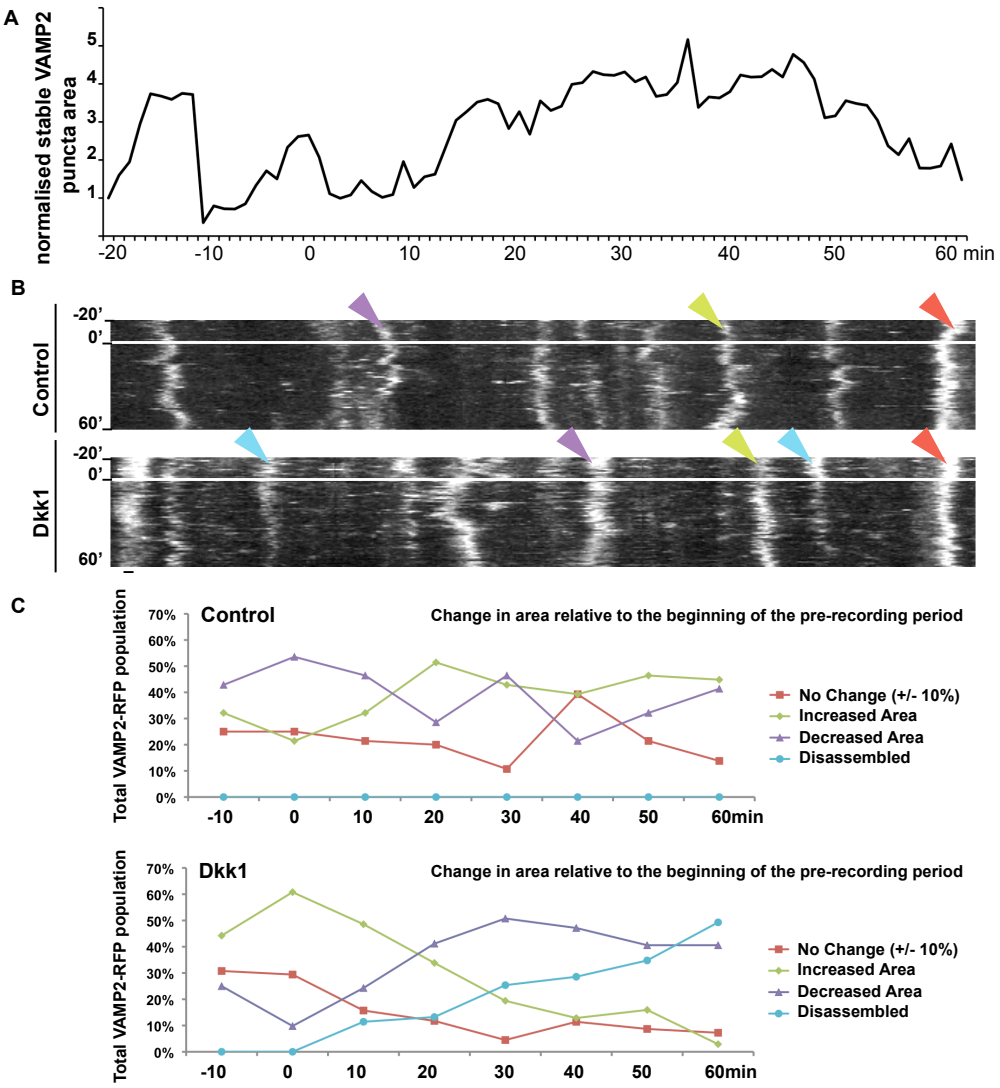


Figure 6.1: Time-lapse recordings reveal that Wnt blockade rapidly alters the dynamic behavior of SV clusters in mature hippocampal neurons. Mature hippocampal neurons expressing VAMP2-mRFP were recorded for 20 min and then Dkk1 or control media was added for 1 hour. Images were acquired at 10 min intervals. (A) Representative plot from a trial experiment illustrates the dynamic changes in puncta area per minute. (B) Kymographs of a single neurite taken from control and Dkk1 treated neurons. The white line indicates where control or Dkk1 media was added. Red arrowheads indicate stable VAMP2-mRFP puncta that persisted throughout the recording period; green arrowheads indicate puncta that increased in size; purple arrowheads indicate puncta that decrease in size; turquoise arrowheads indicate puncta that disassembled. (C) Quantifications show the dynamic behavior of SV clusters is significantly altered in the presence of Dkk1. The graphs depict the behavior of all of the stable SV clusters quantified as a total population. Dkk1 reduced the number of puncta that grew in size, and steadily increased the number of puncta that became smaller compared to controls. In the presence of Dkk1, the number of puncta that disassembled also steadily increased throughout the recording period compared to controls.

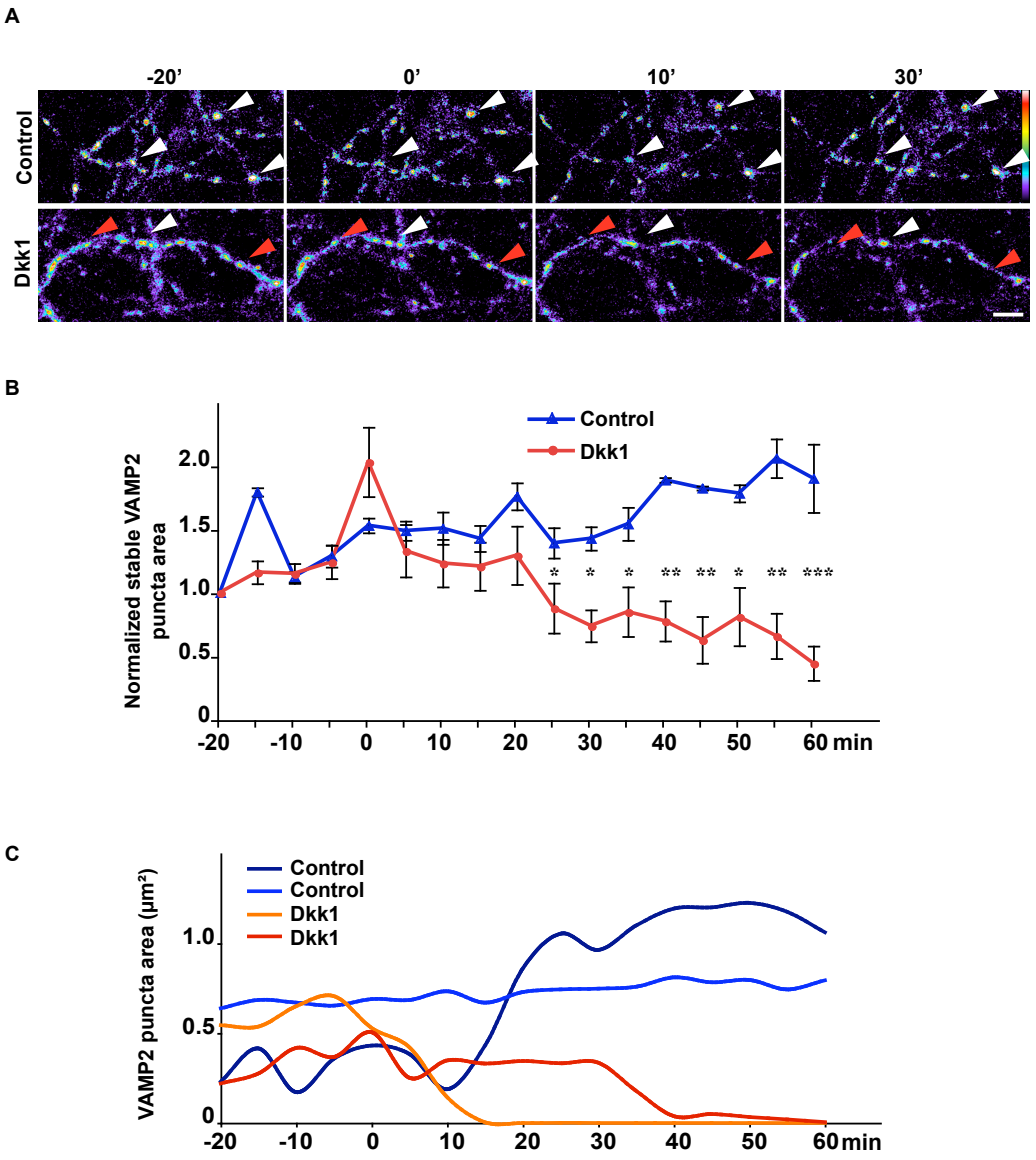


Figure 6.2: Time-lapse recordings reveal that Wnt blockade rapidly induces SV dispersal in mature hippocampal neurons. Mature hippocampal neurons expressing VAMP2-mRFP were recorded for 20 min and then Dkk1 or control media was added for 1 hour. Images were acquired at 10 min intervals. (A) Frames from time-lapse recordings are depicted as pseudo-colour. Images show a decrease in both VAMP2-mRFP puncta area and intensity in the presence of Dkk1 (red arrowheads) indicating SV dispersal from previously stable sites. The images also show stable puncta (white arrowheads). Scale bar, 5 µm. (B) Quantifications reveal that Dkk1 reduced the area of VAMP2-mRFP within 30 min. *p<0.05, **p<0.005, ***p<0.001 ANOVA. Error bars represent SEM. (C) Representative graphs of two individual puncta from control and Dkk1 treated neurons illustrate the typical behavior of puncta size under the two conditions.

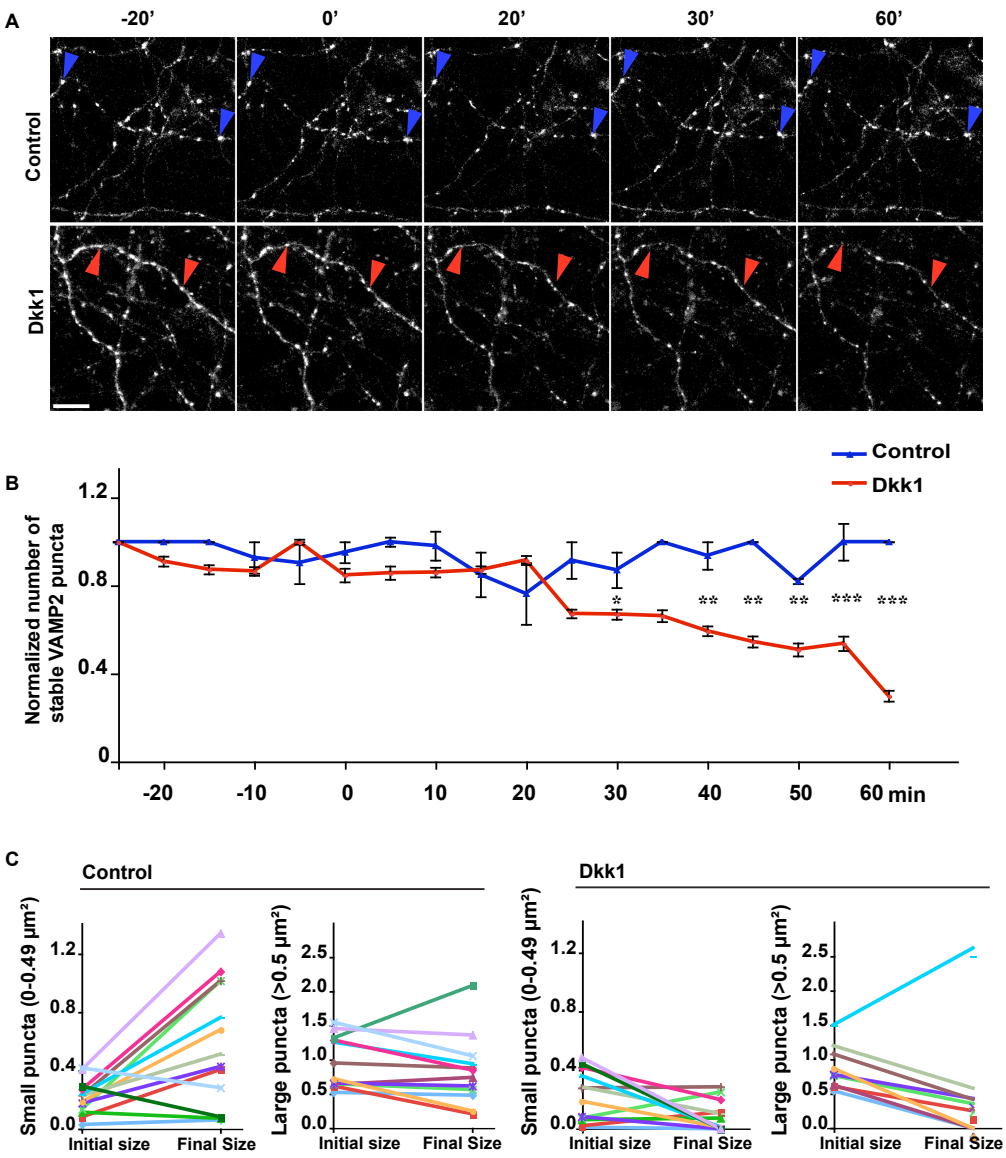


Figure 6.3: Time-lapse recordings reveal that Wnt blockade rapidly induces SV cluster elimination in mature hippocampal neurons. Mature hippocampal neurons expressing VAMP2-mRFP were recorded for 20 min and then Dkk1 or control media was added for 1 hour. Images were acquired at 10 min intervals. (A) Frames from time-lapse recordings illustrate a typical response of stable VAMP2-mRFP puncta under control and Dkk1 conditions. Blue arrowheads indicate stable puncta. In the presence of Dkk1, many stable puncta are lost within the 1-hour recording period. Scale bars, 10 µm. (B) Quantifications revealed that Dkk1 significantly reduced the number of stable VAMP2-mRFP puncta within 30 min compared to control. *p<0.05, ***p<0.001 ANOVA. Error bars represent SEM. (C) Analyses of individual puncta show that both small (<0.49 µm2) and large (> 0.5µm2) VAMP2-mRFP puncta are affected by Dkk1.

clusters according to size, for example, are smaller clusters more susceptible to disassembly? And are larger clusters more resilient? The initial and final sizes of individual puncta were compared and the results show that Dkk1 affects both small (<0.49 μm^2) and large (>0.5 μm^2) SV clusters (Figure 6.3 panel C). Intriguingly, not all SV clusters were affected to the same extent; some of the smaller clusters were unaltered in their size and some clusters even increased in area. However, by the end of the recording period the vast majority of SV clusters were reduced in area and many had been completely eliminated, regardless of their initial size. Interestingly, in the control neurons, smaller SV clusters (<0.49 μm^2) tended to increase in size, whereas larger clusters (>0.5 μm^2) remained constant.

In summary, these data show that Wnt blockade by Dkk1 progressively reduced the intensity and area of VAMP-mRFP clusters to a point where the majority of clusters were completely eliminated. These findings demonstrate that blockade of endogenous Wnt signaling leads to the disassembly and elimination of SV clusters in cultured hippocampal neurons. Importantly, it appears that cluster size does not afford resilience to disassembly as all cluster sizes were affected.

6.3. Discussion

A number of studies have demonstrated that synapses are highly dynamic structures (Holtmaat and Svoboda, 2009). The emerging view now is that synapse formation and elimination contribute to experience-dependent circuit plasticity in the brain (Alvarez and Sabatini, 2007; Holtmaat and Svoboda, 2009). Postsynaptic dendritic spines have the capacity to grow, change morphology or withdraw in response to neural activity (Holtmaat and Svoboda, 2009). Equally, presynaptic SV clusters exhibit remarkably dynamic behaviors; SVs are rapidly mobilized to and from stable cluster sites (Bamji et al., 2006; Kalla et al., 2006; Krueger et al., 2003; Tsuriel et al., 2006), often in an activity-dependent manner (Becker et al., 2008; Frischknecht et al., 2008; Koenig et al., 1993; Li and Murthy, 2001; Staras et al., 2010). Whilst these dynamic cellular behaviors are well documented, the underlying mechanisms that ensure populations of synapses remain intact remain poorly understood.

The data described in Chapter 5 of this thesis suggests that Wnt signaling regulates synapse stability in cultured hippocampal neurons. I found that blockade of endogenous Wnt signaling by Dkk1 induces a loss of large SV clusters with a concomitant gain of small SV clusters suggesting SV dispersal and SV cluster disassembly. Dkk1 significantly reduced the number of presynaptic neurotransmitter release sites and puncta for a range of functionally diverse pre- and postsynaptic proteins. In addition, ultrastructural analyses of remaining synapses revealed shrinkage of the active zone and PSD. Furthermore, co-localization analyses suggested a process of presynaptic disassembly between SVs and cytomatrix proteins in response to Dkk1. Taken together these findings suggest a role for Wnt signaling in maintaining the localization of key synaptic components.

In this chapter I have focused my research on the role of Wnt signaling in the maintenance of stable presynaptic SV clusters, as they are strong indicators of presynaptic presence and function (Ahmari et al., 2000; Alsina et al., 2001; Gomes et al., 2006; Hu et al., 2005; Nonet, 1999; Sabo et al., 2006). Confocal time-lapse microscopy was used to image mature hippocampal neurons transfected with VAMP2-mRFP. The objective was to observe real time SV dynamics of stable clusters during Wnt blockade. The model experiment to demonstrate full synapse disassembly and elimination would be to image the behavior of multiple synaptic proteins during Wnt blockade. However, due to time constraints I limited my assay to the behavior of stable SV clusters. My data shows that under control conditions SV cluster sizes fluctuate considerably within 1-minute intervals. The change in size reflects the addition and removal of SVs and is in accordance with published reports (Bamji et al., 2006; Darcy et al., 2006; Hopf et al., 2002; Kalla et al., 2006; Krueger et al., 2003; Lee et al., 2008b; Tsuriel et al., 2006). I identified four categories of SV cluster behavior; a subset that did not significantly change size, another that increased in size, another that decreased in size and a subset that were eliminated. In control conditions, the percent of the total SV cluster population within the first three categories fluctuated to some extent but remained moderately consistent throughout the recording period. Importantly, no SV clusters were eliminated during the recording period. In contrast, in the presence of Dkk1 the population of stable SV clusters that maintained or increased size was rapidly reduced and the population of clusters that decreased in size concomitantly rose. Significant loss of SV cluster size was observed within 20-30 minutes, which was clearly visualized as a loss of VAMP2-mRFP intensity. Critically, 11% of the stable SV cluster population was eliminated within 10 minutes of exposure to Dkk1, and by 60-minutes over half of all stable cluster sites were lost. My data reveals that Wnt blockade by Dkk1 induces a rapid and progressive loss of SV cluster size prior to complete disassembly. This finding may be consistent with evidence for SV dispersal from stable clusters as is a preliminary event of synapse disassembly (Hopf et al., 2002). However, a detailed time-course for the dispersal/elimination of other synaptic proteins is required to determine a hierarchy of protein disassembly from stable synaptic sites.

Dkk1 is characterized as a robust antagonist of the canonical-Wnt pathway (Niehrs, 2006). Dkk1 binds with the Wnt co-receptor LRP5/6 with high affinity and prevents the Wnt/LRP/Fz complex forming, which effectively blocks canonical-Wnt signaling at the receptor level (Bafico et al., 2001; Mao et al., 2001). Canonical-Wnt signaling acts via the molecular intermediates Gsk3β, β-catenin and TCL/LEF transcription factors to elicit a transcriptional response. Wnt stimulates synapse assembly by a divergent canonical pathway that is independent of transcription (Chapter 3). However, it cannot be assumed that the same pathway regulates synaptic maintenance. Nevertheless, the speed at which Wnt blockade disassembles SV clusters, suggests that Wnts may regulate synapse stability by local mechanisms that are independent from transcription.

In Chapter 4, I demonstrated that inhibition of the serine/threonine kinase Gsk3 with BIO blocked Dkk1 induced SV cluster disassembly. This result suggests that inhibition of Gsk3β is

involved in synaptic maintenance. Gsk3β is enriched at the vertebrate presynaptic bouton (Hirokawa et al., 1989; Landis et al., 1988; Sankaranarayanan et al., 2003) and work from our lab has shown that Gsk3β modulates microtubule dynamics in the axon by phosphorylating microtubule-associated proteins (MAPs) (Ciani et al., 2004), see also (Gould and Brady, 2004). Mutations of *Wnt1/wg* reveal that Wnt signaling at the Drosophila NMJ regulates presynaptic structure and function through cytoskeletal organization via the Gsk3β homologue Shaggy in a divergent canonical Wnt pathway (Ataman et al., 2008). Whilst a role for microtubules regulating SV dynamics or SV cluster stability has yet to be determined, deficits in regulating the presynaptic cytoskeleton may provide a mechanism for Wnt mediated synapse stability.

Actin is enriched at the presynaptic bouton and numerous studies report it as a functional regulator of SV recruitment and release to and from the synapse, as well as maintaining a stable synaptic pool (Cingolani and Goda, 2008). Actin acts as molecular scaffolding and surrounds presynaptic SV clusters (Sankaranarayanan et al., 2003). During synaptic activity, actin monomers are recruited from the axon into the bouton, polymerized and incorporated into the actin network surrounding presynaptic SVs (Sankaranarayanan et al., 2003). Synaptic activity induces the rapid movement of SVs within the presynaptic bouton to ensure efficient neurotransmitter release (see Chapter 1.2 for a detailed discussion of SV dynamics). Therefore, the functional consequence of an actin network surrounding presynaptic SVs may be to prevent dispersal of SVs and other synaptic components during this highly dynamic state. Experiments from our lab show that freezing actin dynamics with latrunculin-A blocks the Dkk1 effect of SV dispersal (unpublished data, Purro and Salinas 2011). This finding raises the question of whether endogenous Wnt signaling regulates actin dynamics in the bouton to maintain the actin scaffolding surrounding SV clusters. Gsk3β is localized to actin-rich domains in the growth cone where it regulates axonal growth (Eickholt et al., 2002). In colorectal tumors, Wnt signaling regulates actin dynamics via APC to affect cell morphology adhesion and migration (Akiyama and Kawasaki, 2006). Whilst it is not clear whether Wnts via Gsk3β or APC regulate actin dynamics within presynaptic boutons of central synapses, such a pathway could provide a model for Wnt mediated maintenance of SV clusters. This avenue of research is being currently pursued in our lab.

The presynaptic scaffolding complex of Piccolo and Bassoon regulates SV clustering and cluster size (Mukherjee et al., 2010; Owald and Sigrist, 2009; Tsuriel et al., 2009), and mice lacking the central domain of Bassoon display synaptic deficits that can be rescued by valproate acid (VPA) (Sgobio et al., 2010). VPA inhibits Gsk indirectly and studies from our lab demonstrate that VPA mimics Wnt signaling in developing neuronal cultures (Hall et al., 2002). Taken together, with my data that reveals Wnt blockade induces dispersal of colocalized SV and Bassoon clusters, and eliminates Bassoon puncta (Chapter 5), these data may reveal a putative link between Wnt signaling via Gsk3β and the presynaptic scaffolding complex that regulates SV clustering. Whilst this link may be tenuous, this may be a candidate pathway to explore Wnt mediated synaptic maintenance.

Wnt signaling acts bi-directionally to regulate synaptic differentiation and function in an activity dependent manner (Ataman et al., 2008). At the *Drosophila* NMJ, activity dependent release of Wnt1/Wg from the bouton induces rapid changes to presynaptic cytoskeletal organization via the Gsk3β homologue Shaggy, and assembles postsynaptic apparatus via the Frizzled nuclear import pathway (Ataman et al., 2008; Packard et al., 2002). At vertebrate central synapses, Wnt isoforms are transcribed and released in an activity dependent manner to regulate dendritic development (Chen et al., 2006; Wayman et al., 2006) and LTP (Chen et al., 2006). Wnt7a regulates presynaptic assembly and neurotransmitter release via Gsk3β (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Hall et al., 2002), and postsynaptic spine growth and strength via CaMKII (Ciani et al., 2011). These studies, which were focused on developing synapses, support a conserved role for Wnt signaling across the synapse and suggest that Wnt signals bi-directionally to regulate vertebrate synapse assembly and function in an activity dependent manner.

Synapses are maintained by activity dependent mechanisms (for a detailed discussion see Chapter 1.3.3). At mature hippocampal synapses, Wnt7a is expressed in response to environmental enrichment, where it regulates synapse assembly (Gogolla et al., 2009). This study suggests that sustained Wnt7a expression is required to maintain newly formed synapses (Gogolla et al., 2009). Here I present time-lapse data that demonstrates Wnt-blockade induces rapid dispersal of SVs from the vast majority of stable SV clusters within 10-20 minutes, which suggests SV clusters are maintained by local Wnt-mediated mechanisms. Importantly, SV cluster shrinkage is associated with complete cluster elimination. Taken together with the immunohistochemical and ultrastructural data (Chapter 5), my results suggest full synapse disassembly and elimination are triggered during Wnt blockade by Dkk1. Possible mechanisms for Wnt mediated synaptic maintenance are via Gsk3β regulation of the cytoskeleton and/or synaptic scaffolding proteins. This report is the first evidence that Wnt signaling regulates SV cluster size and stability in mature hippocampal neurons. Future work will establish the precise mechanisms of Wnt mediated synaptic maintenance.

CHAPTER 7:

7. Discussion

The hippocampus is fundamentally involved in experience/activity-related learning and memory and has become a model system to study the regulation of synapse dynamics during development and maturity. Immunofluorescence against endogenous synaptic proteins is widely used to identify synaptic sites in fixed tissue, and expression of fluorescently tagged synaptic markers has been used in a number of studies to directly visualize synapse assembly and disassembly in live neuronal cultures (Ahmari et al., 2000; Alsina et al., 2001; Gomes et al., 2006; Hu et al., 2005; Nonet, 1999; Sabo et al., 2006). I have used these key-imaging approaches together with electronmicroscopy to study the effects of Wnt signaling on the assembly and disassembly of synapses in hippocampal neurons.

7.1. Summary of results

The role of Wnt signaling molecules as synaptogenic factors has been firmly established for some time (Budnik and Salinas, 2011; Inestrosa and Arenas, 2010; Salinas and Zou, 2008). However, it was not well understood whether Wnt proteins act as pan-synaptogenic factors or if specific isoforms preferentially stimulate the assembly of specific types of synapses. Furthermore, the downstream molecular mechanisms activated during Wnt-mediated synapse assembly had not been fully explored. My studies demonstrate that Wnt7a specifically stimulates the clustering of pre- and postsynaptic proteins for excitatory synapses without affecting the clustering of inhibitory synaptic markers. Importantly, I observed the clustering of several proteins to the same sites, which is a hallmark for the assembly of nascent synaptic sites. To probe for the molecular intermediates that participate in Wnt7a mediated presynaptic assembly, I used specific antagonists against key signaling molecules in the canonical-Wnt pathway. My results reveal that a divergent canonical-Wnt pathway via Gsk3β, that is independent of transcription, stimulates the assembly of excitatory presynaptic sites.

My studies also reveal a novel role for Wnt7a signaling in hippocampal neurons. By using immunohistochemistry against a number of endogenous pre- and postsynaptic proteins in fixed hippocampal cultures, ultrastructural analyses and expression of VAMP2-RFP in live hippocampal cultures, I demonstrate that acute blockade of endogenous Wnt signaling induces rapid synapse disassembly in both young and mature synapses. These data strongly suggest that endogenous Wnts regulate synaptic maintenance by controlling the stability of pre- and postsynaptic sites.

7.2. A role for Wnt signaling in synapse assembly

7.2.1. A divergent canonical-Wnt pathway stimulates presynaptic differentiation of excitatory synapses

The ratio of excitatory and inhibitory synapses is critical to normal brain function. Whilst a number of synaptogenic factors have been identified, little is known about how secreted factors influence the balance of excitation and inhibition in the central nervous system. It has been firmly established that Wnt signaling stimulates the clustering of individual synaptic markers in developing vertebrate and fly neurons highlighting an important role for Wnt-mediated synaptic differentiation (Ahmad-Annuar et al., 2006; Davis et al., 2008; Farias et al., 2009; Gogolla et al., 2009; Hall et al., 2000; Packard et al., 2002). However, evidence for the accumulation of multiple synaptic proteins to single loci and apposition of pre- and post synaptic proteins, which is a conclusive indicator of synapse assembly, has been lacking. In addition, the specificity of Wnt signaling in forming excitatory or inhibitory synapses was not clear. During the writing of this thesis manuscript, research from our lab was published demonstrating a role for Wnt7a mediated assembly of excitatory synapses (Ciani et al., 2011). Critically inhibitory synapses are not affected (Ciani et al., 2011). The data presented in figure 3.2 of this thesis contribute to the published article.

To explore the specific effects of Wnt signaling on the assembly of excitatory synapses *versus* inhibitory synapses, hippocampal cultures were assayed by immunohistochemistry against a number of functionally diverse pre- and postsynaptic synaptic proteins, and proteins that specifically localize to either excitatory or inhibitory synapses. The results presented in this thesis reveal that Wnt7a specifically stimulates the clustering and alignment of pre- and postsynaptic markers that denote excitatory synapses without affecting the clustering or alignment of inhibitory synaptic markers. The assembly of *de novo* synapses was further corroborated by Wnt7a mediated increases in the number of co-localized VAMP2 and Bassoon puncta, which indicates an increase in the number of assembled presynaptic sites, as opposed to an increase in the number of mobile presynaptic transport packets (Ahmari et al., 2000; Friedman et al., 2000; Zhai et al., 2001). My data for the assembly of functional presynaptic sites is in accordance with Wnt-mediated increases in FM1-43 positive puncta, which labels SV recycling at the plasma membrane (Ahmad-Annuar et al., 2006; Cerpa et al., 2008). My data is also consistent with findings from a fellow PhD student in the lab who demonstrated that Wnt7a specifically increases the frequency and amplitude of mEPSPs but not mIPSCs (Ciani et al., 2011). Taken together, my results show that Wnt7a stimulates the assembly of functional excitatory presynaptic sites that are opposed to differentiated postsynaptic sites.

Work from our lab identified Fz5 as the Wnt receptor that regulates presynaptic differentiation (Sahores et al., 2010). Pharmacological inhibition of Gsk3β using lithium, and therefore presumptive activation of the canonical-Wnt pathway, mimics Wnt-mediated presynaptic differentiation (Ahmad-Annuar et al., 2006; Davis et al., 2008; Hall et al., 2000). However,

Wnt7a/b can stimulate presynaptic differentiation within 15 minutes of Wnt application (Ahmad-Annuar et al., 2006), which suggests that the initial stages of Wnt-mediated synapse assembly are independent of transcription. To further examine the underlying mechanisms of Wnt-mediated synapse assembly in the vertebrate CNS, specific antagonists against key molecules within this pathway were used. Dkk1 specifically binds to the Fz co-receptor LRP5/6 with high affinity and prevents the Wnt/Fz/LRP complex forming at the plasma membrane (Mao et al., 2001). Dkk1 completely blocks the ability of Wnt7b to clustering SVs, suggesting a role for LRP5/6 at the plasma membrane for Wnt7a/b mediated presynaptic differentiation. Downstream activation of the canonical-Wnt pathway using the specific Gsk3β antagonist BIO rescued the Dkk1 effect. To test further downstream, I blocked transcription in the presence of Wnt7b. Wnt7b continues to stimulate presynaptic differentiation during transcription blockade. Western blot analyses show that Wnt7a does not induce any significant changes to the level of pre- or postsynaptic proteins, which discounts a role for Wnt-mediated local translation. Together, these findings reveal that LRP5/6 at the receptor level, and Gsk3β at the intracellular level, are involved in regulating Wnt-mediated presynaptic assembly in the absence of transcription. These data reveal that a divergent canonical-Wnt pathway, which bifurcates downstream of Gsk3β, regulates the assembly of excitatory presynaptic sites.

My studies, and those from our lab have focused on the role of Wnt7a on excitatory synapse assembly. However, it is important to note that other Wnt isoforms may regulate synaptogenesis with less specificity; for example, Wnt5a has been reported as a regulatory factor in the assembly of excitatory and inhibitory synapses (Cuitino et al., 2010; Farias et al., 2009; Paina et al., 2011). The studies on Wnt7a and Wnt5a indicate that while Wnt7a specifically promotes excitatory synapses, Wnt5a seems to function as a pan-synaptogenic factor. A systematic analysis of Wnt isoform activity on the specification of synapse type has yet to be completed.

Work from our lab demonstrates a divergent-canonical pathway regulates microtubule stability via Gskβ phosphorylation of MAP1B (Ciani et al., 2004; Lucas and Salinas, 1997). Furthermore, a divergent-canonical pathway regulates microtubule directionality and organization in the growth cones of cultured mouse dorsal root ganglion cells during axon guidance (Purro et al., 2008). Thus, a divergent canonical Wnt signalling pathway regulates axon remodeling. These findings raise the question whether a similar mechanism may regulate Wnt7a/b mediated presynaptic assembly. My experiments demonstrated that Wnt7a/b does not alter the levels of key pre- and postsynaptic proteins. Moreover, this effect is not affected by blockade of transcription. In contrast, Wnt7a does regulate the distribution of synaptic components. Indeed, Wnt7a decreases the number of small clusters of SVs with a concomitant increase in the number of larger clusters. The shift in SV cluster size may represent the capture and aggregation of small mobile SV clusters or SVTs, possibly by local reorganization of the cytoskeleton, which could traffic molecules to nascent synaptic sites. If this proposition is correct, then PVTs could also be ‘captured” at the same sites.

Synaptic tagging is a hypothesis to explain how individual synapses are selectively modified according to previous synaptic activity (Frey and Morris, 1997). Whilst synaptic tagging has

been principally studied in postsynaptic dendrites and spines during LTP and LTD, it is possible that similar mechanisms regulate selective delivery of synaptic proteins to nascent synapses. The premise for the synaptic "tag" theory is that plasticity related proteins (PRPs) are transported via the cytoskeleton to activated synapses to modulate long lasting plasticity; neighboring unstimulated synapses do not receive the same cargos (Barco et al., 2008; Frey and Morris, 1997; Martin and Kosik, 2002). This suggests that individual synapses are "tagged" and labeled as targets for PRP recruitment. Whilst the precise nature of synaptic "tags" has not been fully defined, it is most likely to depend upon multiple molecular strategies that include changes to the cytoskeleton (Maas et al., 2009; Martin and Kosik, 2002; Okamoto et al., 2009; Ramachandran and Frey, 2009). Activity dependant mechanisms induce localized modifications to microtubules at the base of spines via changes in microtubule-associated proteins (MAPs), which direct PRPs into spines (Maas et al., 2009). In addition, the F-actin network, which is modified via CaMKII activity (Okamoto et al., 2009), is directly involved in a tag specific molecular complex in apical CA1 dendrites and contributes to the capture process of PRPs (Ramachandran and Frey, 2009) and PRP recruitment into the PSD and perisynaptic regions (Chen et al., 2007; Fukazawa et al., 2003; Lisman, 2003; Peng et al., 2010; Shoji-Kasai et al., 2007; Yang et al., 2008). Studies from our lab and others have shown that Wnt signaling regulates both microtubule and actin dynamics, as well as CaMKII activity (Akiyama and Kawasaki, 2006; Ciani et al., 2011; Ciani et al., 2004; Purro et al., 2008). It is therefore tempting to speculate that Wnt-dependant local changes to microtubule and actin organization and dynamics in the pre- and postsynaptic terminals may serve as traffic signals to direct SVs, SVTs, PVTs and postsynaptic proteins to nascent synapses. A possible role for Wnts in synaptic tagging remains to be explored.

7.2.2. Bidirectional Wnt signaling regulates excitatory synapse assembly

My experiments demonstrate that Wnt7a/b stimulates SV clustering via a divergent canonical-Wnt pathway in isolated axons in young hippocampal cultures (5-7 DIV) plated at very low density. This suggests that Wnt7a/b signals directly to the axon to regulate presynaptic differentiation. Consistent with this view, mossy fiber axons deficient in Dvl1 do not respond to Wnt7a/b (Ahmad-Annuar et al., 2006). Wnt7a/b also stimulates the recruitment and accumulation of key presynaptic proteins and regulates SV cycling and glutamate release probability (Ahmad-Annuar et al., 2006; Cerpa et al., 2008). Work from our lab also reveals that Wnt7a signals directly to the postsynaptic dendrite, through activation of CaMKII, to regulate spine morphology, function and maturity (Ciani et al., 2011). In this study, exogenous Wnt7a or overexpression of postsynaptic Dvl1 increases the frequency and amplitude of miniature EPSCs. Conversely, dendrites from Dvl1 mutant neurons do not respond to Wnt7a. Furthermore, Wnt7a:Dvl1 mutant mice display significant deficits in spine morphology and synaptic transmission, which reveals a role for endogenous postsynaptic Wnt signaling regulating synaptic development and strength. Bath application of Wnt7a increases the number of spines, whereas postsynaptic activation of Wnt signaling regulates spine morphology,

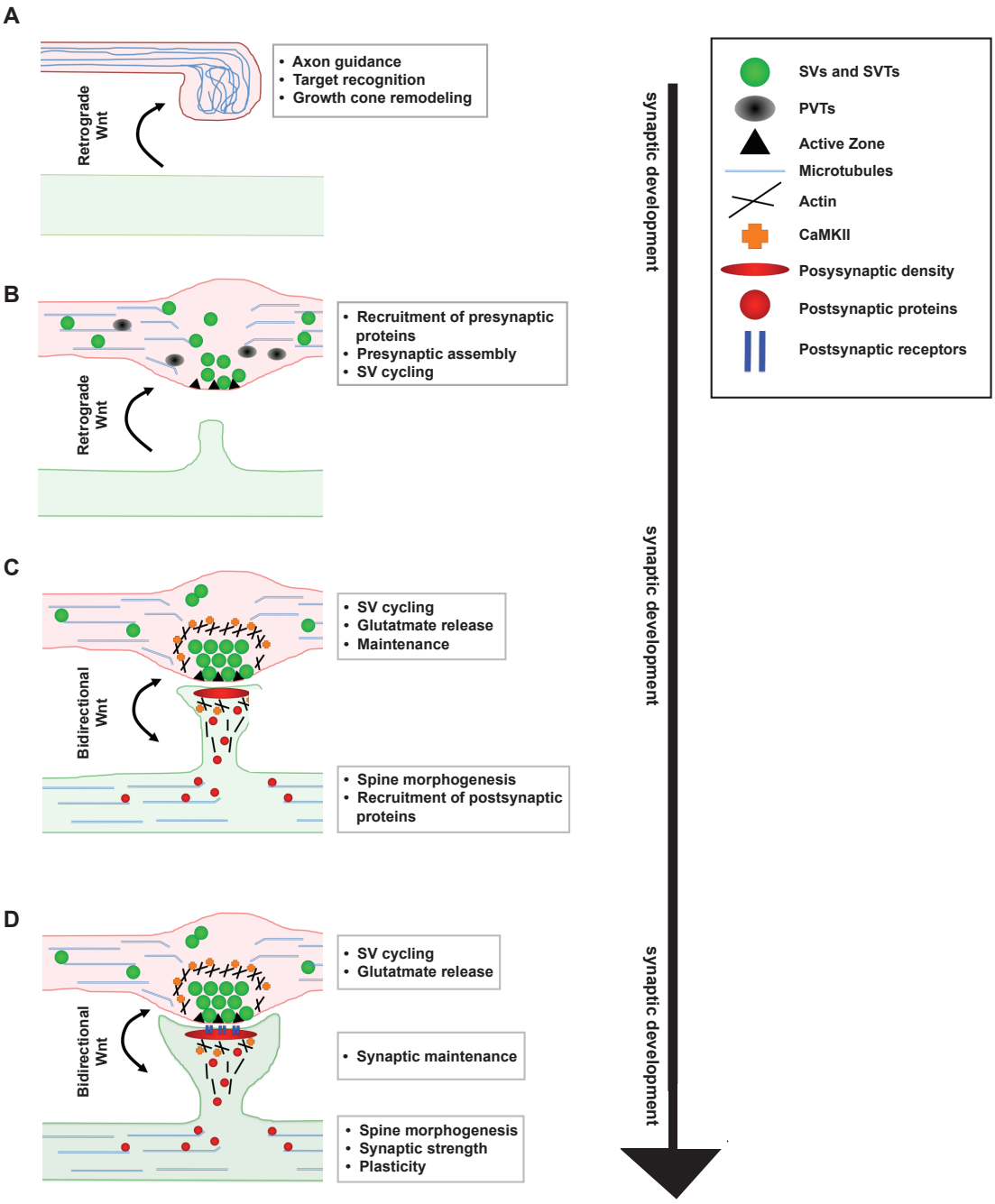


Figure 7.1. A model for Wnt regulation of synapse development, maturity and maintenance. The illustration outlines key phases of Wnt mediated synapse development. A) Wnt regulates axon guidance in the peripheral and central nervous systems. Target derived Wnt signals guide incoming axons and induce axon and growth cone remodeling by modulating the cytoskeleton. B) Wnt signals directly to the axon to stimulate the recruitment of presynaptic proteins and regulate presynaptic assembly. Wnts also regulate SV cycling in the presynaptic bouton. C) Bidirectional Wnt signaling continues to modulate SV cycling and neurotransmitter release in the presynaptic bouton. Wnt also signals directly to the postsynaptic dendrite to stimulate spine morphogenesis, postsynaptic protein recruitment and synaptic strength. D) Wnts are expressed and released from mature synapses in an activity dependent manner. Wnt signaling continues to regulate SV cycling and neurotransmitter release at mature synapses and regulates synaptic maintenance. In the presynaptic terminal, Wnts signal via a divergent canonical pathway to stimulate synapse assembly and synaptic maintenance. Post-synaptically, Wnt signals via CaMKII to regulate spine morphogenesis and synaptic strength.

maturity and function without affecting spine density (Ciani et al., 2011). This suggests that Wnt7a may stimulate the initial phases of spine formation indirectly through presynaptic assembly and/or activity. Subsequently, postsynaptic Wnt signaling through Dvl1 and CaMKII regulates spine morphology, maturation and function (Figure 7.1). Taken together, the evidence supports a model for bidirectional Wnt7a/b signaling to regulate the assembly and function of excitatory synapses.

A model for bi-directional Wnt signaling with different pathways in the pre- and postsynaptic terminals has been proposed to regulate formation and function of *Drosophila* NMJs (Ataman et al., 2008). In this model, the authors present evidence for activity dependent Wg release from presynaptic boutons. A divergent canonical-Wg pathway, via Shaggy (the *Drosophila* Gskβ homologue) and phosphorylation of Fusch (the *Drosophila* MAP1B homologue) regulates presynaptic microtubule dynamics, which modulate structural development of the bouton (Ataman et al., 2008). In addition, in the postsynaptic muscle, Wingless signals through the Frizzled nuclear import pathway to promote postsynaptic differentiation (Ataman et al., 2008). Whilst there are differences in the underlying mechanisms of Wnt signal transduction between the fly and mouse models, there are important similarities that indicate conservation across the species in terms of bidirectional Wnt signaling in the regulation of synaptogenesis. In particular, the role of a divergent canonical-Wnt/wg pathway via Gsk3β/shaggy that is independent of transcription in the assembly of presynaptic terminals.

In summary, my findings report that Wnt7a directly stimulates differentiation of excitatory presynaptic sites through the activation of a divergent canonical-Wnt pathway in the presynaptic side, which is accompanied by the concomitant postsynaptic differentiation. Importantly, inhibitory synapse formation was unaffected, which concludes that Wnt7a specifically stimulates the assembly of excitatory synapses. Recently published data from our lab demonstrates that Wnt7a signals directly to the postsynaptic dendrite to stimulate spine morphogenesis, maturity and synaptic strength (Ciani et al., 2011). Together, these results provide novel insights into the role of bidirectional Wnt signaling at central vertebrate synapse. Further work is required to determine the specificity of other Wnt isoforms in terms of their capacity to stimulate excitatory and/or inhibitory synapses. Future studies will elucidate the precise mechanisms of Wnt7a mediated presynaptic differentiation and determine whether modulation of the cytoskeleton is key for synaptic assembly.

7.3. A role for Wnt signaling in normal brain function

7.3.1.Wnt-mediated synaptic plasticity

A role for activity dependent Wnt expression and release with functional outcomes has been demonstrated in the developing and mature brain. Neuronal activity stimulates Wnt expression and release (Wayman et al., 2006; Yu and Malenka, 2003), where it regulates dendrite development and arborization (Rosso et al., 2005). Importantly, neuronal activity stimulates the

clustering of Fz receptors to the cell surface at synaptic sites, which enables neurons to respond to secreted Wnts and activate intracellular signaling cascades (Sahores et al., 2010). Later in development, and in the adult hippocampus, neuronal activity stimulates the release of Wnts (Chen et al., 2006; Gogolla et al., 2009), where it stimulates synaptogenesis, synapse remodeling and LTP (Chen et al., 2006; Gogolla et al., 2009; Sahores et al., 2010). Presynaptic activation of Wnt signaling increases the frequency of spontaneous and miniature EPSCs, which represents enhanced release probability (Avila et al., 2010; Beaumont et al., 2007; Cerpa et al., 2008). Critically, mice deficient in Wnt7a/Dvl1 display deficits in neurotransmitter release (Ahmad-Annuar et al., 2006; Ciani et al., 2011). Postsynaptic activation of Wnt signaling regulates spine morphology and synaptic strength (Ciani et al., 2011). Collectively, these studies support a model for Wnt signaling on both sides of the vertebrate synapse and present a persuasive case for activity dependent Wnt-mediated synaptic plasticity.

In addition to promoting presynaptic differentiation, Wnts promote the recruitment of synaptic proteins and SVs to nascent synaptic sites, my studies suggest that Wnt7a/b might stimulate the recruitment of SVs to existing active synapses as suggested by an increase in SV cluster area. It has been suggested that the recruitment of SVs to existing presynaptic sites presents a mechanism for regulating presynaptic strength by expanding the recycling and resting pool, and altering release probability (Darcy et al., 2006; Murthy et al., 1997; Staras et al., 2010). Importantly, the newly recruited SVs participate in neurotransmitter release (Staras et al., 2010). LTP induces the rapid recruitment of the SV associated protein synaptophysin to presynaptic sites (Antonova et al., 2001), which implies an increase in SV cluster size by activity dependent mechanisms. Future studies focused on the recruitment of SVs to stimulated synapses will determine if activity dependent release of Wnt plays a role in expanding the SV population, and whether these SVs are incorporated into the recycling pool to facilitate presynaptic plasticity.

7.3.2. Endogenous Wnt signaling regulates synapse stability

Science is full of serendipity, and my share came with the observation that blockade of Wnt signaling by Dkk1, in the absence of exogenous Wnt7b, induced a significant and rapid loss of SV clusters. It was not logical to reason that blockade of synaptogenesis could account for ~50% loss of SV clusters compared to control cells. Rather, a more reconcilable interpretation was that Dkk1 induces the loss of existing SV clusters, which would reveal a novel role for Wnt signaling in synaptic maintenance. Based on this supposition, my PhD thesis changed track and I began to probe for Wnt-mediated synapse disassembly, rather than Wnt-mediated synapse assembly.

SV clusters are highly mobile and are transported along neurites and incorporated into new and/or existing synaptic sites (Ahmari et al., 2000; Darcy et al., 2006; Hopf et al., 2002). The availability of mobile extrasynaptic SVs is believed to underscore the rapid assembly of synapses (Ahmari and Smith, 2002; Matteoli et al., 2004; Ziv and Garner, 2004), and their dispersal from stable, functional presynaptic sites is an early event in synapse disassembly

(Hopf et al., 2002). SVs shuttle between adjacent synapses and constitutive sharing of SVs between neighboring synapses has been reported as a potential homeostatic mechanism that enables neighboring synapses to distribute synaptic weights and locally coordinate release probabilities (Darcy et al., 2006). The dispersal and incorporation of mobile SVs and synaptic proteins from and into active synapses underscores changes to the efficacy of synaptic transmission and presynaptic remodeling associated with long-term synaptic plasticity (Antonova et al., 2001; Zakharenko et al., 2001). These studies also underscore the need for mechanisms to ensure a persistent pool of SVs is available at active synaptic sites.

My time-lapse experiments and analyses are consistent with other experiments that reveal mobile SVs are trafficked along neurites (Ahmari et al., 2000; Darcy et al., 2006; Friedman et al., 2000; Hopf et al., 2002; Lee et al., 2008b). Over a 100-minute recording period, I found that ~60% of VAMP2-RFP puncta were in transit in 21-23 DIV hippocampal neurons. Analyses of stable puncta size was made every 10 minutes and I found only ~25-30% remained within ±10% of there original size at any one time point under control conditions. Stable SV clusters rapidly grew and shrank in size, which is consistent with the notion of SVs being shuttled between synapses and recycled (Darcy et al., 2006). Remarkably, these changes could be observed in real time, as could the shuttling of SVs along the neurite. The rapid fluctuations in SV cluster size and mobility reflect the dynamic state of presynaptic SV pools and underscore the requirement for mechanisms to maintain synaptic structure.

Blockade of Wnt signaling with Dkk1 induces a rapid loss of SV cluster sites. Under control conditions, none of the stable SV puncta selected for analysis disassembled. In contrast, stable SV clusters disassembled within 10-minites of Wnt blockade, and the number of disassembled SV clusters steadily increased throughout the recording period. Furthermore, Wnt blockade induced a steady increase in the population of SVs that decreased in size, and fewer SV clusters increased in size. In the fixed-tissue immunofluorescence experiments, Dkk1 induces a clear and significant shift in SV cluster size towards smaller puncta with a concomitant loss of larger puncta. The increase in very small puncta may reflect SVs dispersing from stable sites. Indeed, during the time-lapse imaging, I observed tiny clusters scattering from larger stable sites. The quantified changes suggest that Wnt blockade induces synapse elimination by a process of SV dispersal.

In the presence of Dkk1, the loss of SV clusters was associated with a significant decrease in the number of puncta for pre- and postsynaptic protein markers such as Bassoon, Cask, Synapsin1, PSD95 and Neurologin2 suggesting full synapse disassembly and elimination within 2-hours. If only one or two markers had been affected, it could suggest that Wnt signaling modulates a sub-set of synaptic proteins at the synapse, or certain structures by regulating the reserve or readily releasable pools of SVs, or elements of the active zone. However, this was not the case. Rapid synapse elimination was further conferred by a loss of neurotransmitter release sites detected by FM1-43 labeling, and by an increase in de-localization of two presynaptic markers VAMP2/Bassoon suggesting presynaptic disassembly. Western blots revealed no significant loss of synaptic proteins, which supports the notion that synaptic

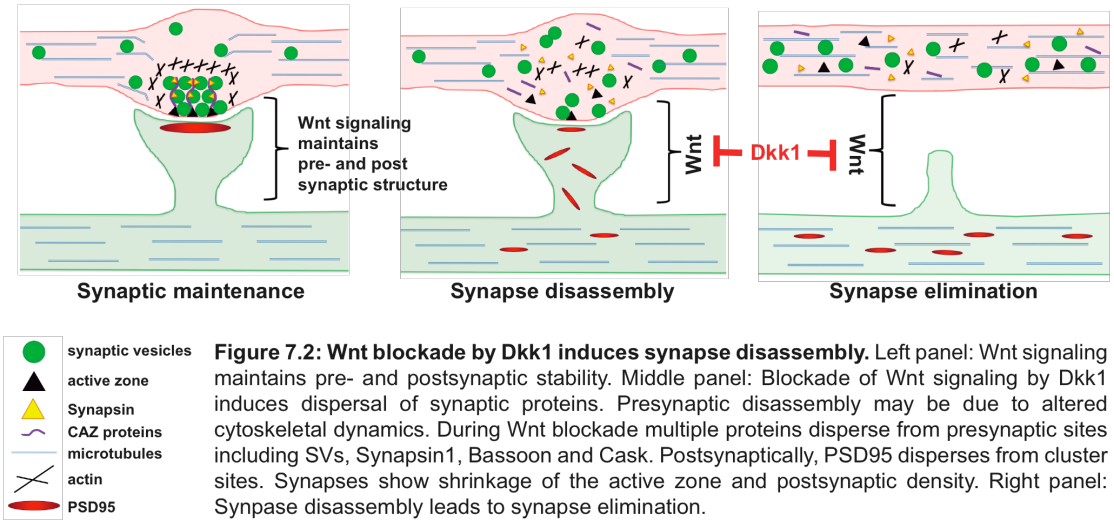


Figure 7.2: Wnt blockade by Dkk1 induces synapse disassembly. Left panel: Wnt signaling maintains pre- and postsynaptic stability. Middle panel: Blockade of Wnt signaling by Dkk1 induces dispersal of synaptic proteins. Presynaptic disassembly may be due to altered cytoskeletal dynamics. During Wnt blockade multiple proteins disperse from presynaptic sites including SVs, Synapsin1, Bassoon and Cask. Postsynaptically, PSD95 disperses from cluster sites. Synapses show shrinkage of the active zone and postsynaptic density. Right panel: Synpase disassembly leads to synapse elimination.

proteins were dispersing from stable, functional synaptic sites, rather than being degraded Ultrastructural analyses revealed that short-term Wnt blockade significantly reduced the length of both the AZ and PSD at remaining synapses. It is interesting to note that the AZ and PSD are size matched in both control and Dkk1-treated neurons, which is consistent with other ultrastructural analyses for coordinated pre- and postsynaptic changes in size (Harris et al., 1992) and suggests the presence of a molecular dialogue coordinating disassembly of pre- and postsynaptic compartments, which is consistent with a role for bidirectional Wnt signaling across the synapse. Together, my results suggest a process of synapse disassembly, with a loss of pre- and postsynaptic scaffolding proteins, active zone material and SVs when Wnt-signaling is blocked (Figure 7.2).

An enduring message that I hope to have conveyed throughout this manuscript is that SV clusters and synapses are highly dynamic structures. SVs continually cycle within the presynaptic bouton and during intense action potential stimulation, SVs are mobilized from the reserve and resting pools to participate in neurotransmitter release at the AZ (Fdez and Hilfiker, 2006; Rizzoli and Betz, 2005; Sudhof, 2004; Sudhof and Rothman, 2009). Furthermore, SVs, synapsins and actin transiently disperse from the AZ region during presynaptic stimulation, and then subsequently regroup (Chi et al., 2001; Sankaranarayanan et al., 2003; Tao-Cheng, 2006a). This raises the question of whether Wnt blockade induces SV dispersal resulting in synapse disassembly, or prevents SV reclustering and subsequent incorporation into stable clusters following stimulation.

A recently proposed model suggests a proteinaceous inter-vesicular matrix (IVM) maintains SV clusters at resting conditions and restricts SV mobility within the AZ region (Pechstein and Shupliakov, 2010). The precise composition of the IVM remains unknown but it is believed to be partly composed of endocytic proteins that are dephosphorylated upon Ca^{++} influx into the presynaptic bouton. Changes in the phosphorylation state of the IVM is believed to destabilize

the cytoskeletal matrix enabling SV movement within the cluster to fulfill the role of activity-dependent neurotransmitter release, and permits SV dispersal from the cluster into the perisynaptic region and axon (Pechstein and Shupliakov, 2010). In addition, to the IVM that permeates SV clusters the model includes an actin "cage" that surrounds SV clusters (Sankaranarayanan et al., 2003). It is suggested that the stability of this actin "cage" is regulated by CaMKII activity (Tao-Cheng et al., 2006b), which in turn is regulated by Ca^{++} influx during presynaptic activity. Work from our lab has recently demonstrated a role for Wnt7a/Dvl1 signaling in the activation of CaMKII in postsynaptic spines (Ciani et al., 2011). It would therefore be important to understand if Wnt7a equally activates presynaptic CaMKII to regulate actin dynamics and the integrity of the cage it forms surrounding stable SV clusters. To return to the question of whether Wnt blockade induces disassembly, or prevents the reassembly or synaptic proteins following activity-dependent dispersal; it is tempting to speculate that Wnt signaling through CaMKII may be involved in maintaining the actin cage that maintains SV clusters at rest, and may possibly provide a synaptic "tag" (as previously described) for the reclustering of SVs and other dispersed presynaptic proteins. Therefore, in the absence of Wnt signaling, SVs and synaptic proteins may disperse and be unable to regroup following activity dependent dispersal.

Inhibition of Gsk3 by the specific inhibitor, BIO, rescued the loss of SV clusters induced by Dkk1 further supporting a role for the canonical pathway. However, the effects of Wnt7a/b and Dkk1 on SV clusters are independent of transcription. These data suggest that activation of a divergent canonical-Wnt pathway is involved in maintaining SV clusters at synaptic sites. Gsk3 regulates actin and microtubule dynamics (Ciani and Salinas, 2005; Eickholt et al., 2002; Ikeda et al., 1998). Disruption of the cytoskeletal structure and/or dynamics by Wnt/Gsk3β signaling could potentially enable SV and synaptic protein dispersal by disturbing structural boundaries and prevent reclustering. Alternatively, Gsk3β activity could be involved in stabilizing or destabilizing the IVM.

Without a sequential profile of disassembly events, in terms of the order in which synaptic components are modulated or dispersed from synapses during Wnt blockade, it is difficult to hypothesize the precise nature of Wnt mediated synaptic maintenance. Could modulation of the cytoskeleton permit dispersal of synaptic proteins, either at rest or prevent their recapture following stimulation? Could SV dispersal trigger dissolution of the AZ? Or could disruption of the AZ or mechanisms that regulate SV cycling induce SV dispersal? Given the rapidity of Dkk1 induced SV dispersal, it could be reasonable to speculate that SV dispersal is one of the earliest events, especially considering that Bassoon is believed to be a very stable synaptic component. However, changes in Bassoon's ability for acting as a scaffold protein could potentially precipitate SV dispersal. Further research into the hierarchy of Dkk1 induced disassembly, the role of the cytoskeleton and understanding Wnt mediated interactions between synaptic proteins are required to fully elucidate how Wnt blockade induces rapid synapse disassembly and elimination.

7.3.3.Wnt as a modulator of synapse assembly, maintenance and disassembly

As discussed, Wnt signaling stimulates synapse assembly in the developing and mature hippocampus and modulates synapse function (Budnik and Salinas, 2011; Ciani et al., 2011; Gogolla et al., 2009; Salinas and Zou, 2008). An ongoing requirement for neuronal Wnt signaling is consistent with reports of a dynamic yet persistent expression of Wnt in the vertebrate CNS from early embryonic stages through to adulthood (Coyle-Rink et al., 2002; Davis et al., 2008; Gogolla et al., 2009; Lucas and Salinas, 1997; Rosso et al., 2005; Shimogori et al., 2004). To date, BDNF is the only protein that has been reported to mediate synapse assembly, function and maintenance, and link these critical processes with neuronal activity (Cunha et al., 2010; Hu et al., 2005). My studies demonstrate a novel role of Wnt mediated synaptic maintenance, which like BDNF extends its role beyond synapse assembly and function.

Synapse assembly, function and maintenance are activity dependant processes (Becker et al., 2008; De Paola et al., 2003; Holtmaat and Svoboda, 2009; Hopf et al., 2002; Katz and Shatz, 1996; Tanaka et al., 2000). Neuronal activity stimulates synaptogenesis and synapse function through Wnt dependent mechanisms in the developing and mature hippocampus (Chen et al., 2006; Gogolla et al., 2009; Sahores et al., 2010). Taken together with my findings, it is thus reasonable to speculate that Wnt signaling may also be a mechanism for activity dependent synaptic maintenance. This surmise could reveal a mechanism that links activity dependent synapse assembly, function and maintenance with ongoing Wnt expression in the brain. Further studies focused on activity dependent Wnt release and its effects on synapse stability are required to examine this conjecture. Furthermore, understanding the mechanism underlying synaptic maintenance have important implications for translational research into the loss of synapses that hallmark neurodegenerative diseases such as Alzheimer's disease which are hallmarked by a catastrophic loss of synapses.

7.4. A role for Wnt in the diseased brain

The role of Wnt signaling in cancer biology is firmly recognized (Clevers, 2006; Klaus and Birchmeier, 2008; MacDonald et al., 2009) and research continues to be published at a prolific rate. In addition to this well-established role, Wnt signaling is emerging as an important player in the neurodegenerative disorders Alzheimer's disease (AD) and Parkinson's disease (PD) and neurodevelopmental disorders such as autism and schizophrenia (Inestrosa and Arenas, 2010){De Ferrari, 2006 #1011).

7.4.1.Neurodevelopment disorders

The results presented in this thesis suggest that Wnt7a could affect the ratio of excitatory and inhibitory (E/I) transmission in the developing brain. E/I imbalances are implicated in

neurodevelopment disorders such as epilepsy, autism, schizophrenia and Rett syndrome {Medrihan, 2008 #1019}(Kehrer et al., 2008; Munoz-Yunta et al., 2008; Rubenstein and Merzenich, 2003). Whilst evidence for Wnt-mediated changes in E/I transmission in such neurodevelopment disorders has not been published, a role for Wnt signaling is emerging as a research focus in understanding the etiology of such neurologic disorders (De Ferrari and Moon, 2006; Inestrosa and Arenas, 2010). For example, in the autistic brain, Wnt signaling mediates the overgrowth of brain tissue structures that are believed to contribute to autism pathology (De Ferrari and Moon, 2006), and *Wnt2* genetic mutations have been discovered as autism susceptibility markers (De Ferrari and Moon, 2006).

Schizophrenia

A hypothesis for cellular mechanisms underlying peripubescent schizophrenia includes defects in synaptic pruning (Feinberg, 1982; Hayashi-Takagi et al., 2011). In the normal adolescent brain, it is believed that significant numbers of synapses are eliminated in a process that "reorganizes" neural circuits (Feinberg, 1982; Feinberg et al., 1990). In biopsied brains of schizophrenic individuals, there is evidence of excessive synaptic pruning (Feinberg, 1982; Hayashi-Takagi et al., 2011), which may be due to defects in synaptic maintenance (Hayashi-Takagi et al., 2011; Hayashi-Takagi and Sawa, 2010). It has also been suggested that insufficient synaptic pruning may underlie some of the psychiatric disorders displayed by schizophrenic patients (Feinberg, 1982). Dysregulated Wnt signaling has been detected in the CA3 and CA4 regions of the hippocampus in post-mortem schizophrenic individuals, which include elevated expression of Wnt1 (Miyaoka et al., 1999) and reduced cytoplasmic β-catenin (Cotter et al., 1998). Whilst there is no direct evidence for Wnt signaling participating in synapse pruning in the normal or schizophrenic adolescent brain, it is interesting to speculate that altered Wnt signaling could interfere with synapse pruning through its role as a synaptogenic and/or maintenance factor. Dkk1 is a target gene of Wnt signaling (Gonzalez-Sancho et al., 2005; Niida et al., 2004); it would therefore be interesting to examine whether enhanced Dkk1 expression, due to elevated Wnt1 levels, is associated in these regions, which could elucidate a mechanism for excessive synapse loss.

7.4.2. Neurodegenerative disorders

Parkinson's disease

Parkinson's disease (PD) is associated with progressive synapse dysfunction and synapse loss (Hashimoto et al., 2003; Jellinger, 1996; LaFerla and Oddo, 2005). A common mutation found in PD patients is found within the PARK8 gene, which encodes the leucine-rich-repeat cytosolic protein kinase LRRK2 (Sancho et al., 2009). The common PARK8 missense mutation is understood to be causative in PD neuronal cell death, but the downstream mechanisms remain poorly understood (Greggio et al., 2006; West et al., 2007). It was recently discovered that

LRRK2 binds with Dvl1, and these interactions are weakened in the mutated LRRK2 (Sancho et al., 2009). Dvl1 regulates microtubule stability in a divergent canonical-Wnt pathway (Ciani et al., 2004). LRRK2 colocalizes with Dvl1 in neurites, where it is thought to cooperate in microtubule stability (Sancho et al., 2009). Consequently, it has been suggested that LRRK2 mutations may interfere with Wnt/Dvl mediated microtubule stability, which could reveal a mechanism for neurite and synapse dysfunction, and based upon my data, a possible cause for synapse disassembly. Wnt signaling has also been implicated in dopaminergic neurogenesis, cell survival, neurite branching and synaptogenesis (Inestrosa and Arenas, 2010) and it is plausible that Wnts regulate neurotransmitter release as it does in the cerebellum and hippocampus (Ahmad-Annuar et al., 2006; Cerpa et al., 2008). Whilst the evidence is still tenuous for Wnt mediated synapse dysfunction and synapse loss in PD, further research focused on Wnt signaling in the striatum will determine whether dysregulated Wnt signaling contributes to PD pathology.

Alzheimer's disease

Alzheimer's disease (AD) is complex neurodegenerative disorder characterized by chronic progressive decline of neural metabolism, morphology and function, with associated loss of cognitive function. It is estimated that 4.5 million people will develop AD every year, and by 2050 the projected number of AD suffers is 115 million worldwide (Zetzsche et al., 2010). Autosomal dominant familial AD (FAD) accounts for ~5% all AD cases and is associated mainly with early onset AD (EOAD), the remaining cases are sporadic and are associated primarily with late onset AD (LOAD) (Bertram and Tanzi, 2009). The genetic etiologies of FAD and sporadic AD are quite distinct; FAD is caused by highly penetrative mutations in three genes (*APP, PSEN1* and *PSEN2*), which are involved in Amyloid-β production (Aβ). In contrast, over 200 risk genes are associated with sporadic LOAD (Avramopoulos, 2009). Within this large number of risk genes, a core have been identified that are involved in Aβ, lipid and chaperone production and metabolism, as well as immune response and chronic inflammatory mechanisms (Zetzsche et al., 2010). However, considerable research is required to understand how these risk genes directly impact on synaptic and/or neuronal function. Over expression of the complement gene *C1q*, which is a part of the innate immune system, has been identified in the AD brain (Fonseca et al., 2004; Walker and McGeer, 1992). C1q protein mediates developmentally regulated synapse disassembly in the visual system (Stevens et al., 2007) and its upregulation in the AD brain provides insights into mechanisms underlying the widespread loss of synapses that hallmark this disorder.

A role for Wnt signaling in the pathogenesis of AD has been suggested by a number of studies (Caraci et al., 2008; De Ferrari and Moon, 2006; Inestrosa and Arenas, 2010). Soluble Aβ binds to Fz5 and blocks canonical-Wnt signaling (Magdesian et al., 2008). A genetic variant of LRP6, the receptor for Dkk1, is associated with late-onset AD (De Ferrari et al., 2007), and aberrant Gsk3β activities hyperphosphorylate Tau (Hooper et al., 2008) and adversely affect amyloid precursor protein processing (Mudher et al., 2001). Furthermore, exogenous Wnt can

ameliorate Aβ-induced neurodegeneration (Cerpa et al., 2010; De Ferrari et al., 2003). Dkk1 expression is elevated in human postmortem AD brains and mouse models of AD (Caricasole et al., 2004; Rosi et al., 2010), and Dkk1 has been described as a trigger for neuronal death in neurodegenerative conditions including AD, ischemia and epilepsy (Busceti et al., 2007; Cappuccio et al., 2005; Caricasole et al., 2004; Mastroiacovo et al., 2009; Rosi et al., 2010; Scali et al., 2006).

Synapse loss often precedes the catastrophic neuronal cell death that hallmarks AD, and other neurodegenerative diseases including Parkinson's, glaucoma and amyotrophic lateral sclerosis (Luo and O'Leary, 2005; Saxena and Caroni, 2007; Selkoe, 2002; Stevens et al., 2007). This observation implies synapse disassembly is an early event in neurodegenerative disease. Dkk1 expression has been suggested as causative of the neuronal death that underlies AD (Caricasole et al., 2004; Rosi et al., 2010). However, my analyses from both TUNEL and recovery experiments in cultured hippocampal neurons reveal no evidence of Dkk1 induced neuronal death within 24 hours of Dkk1 exposure. Furthermore, studies from our lab of transgenic mice expressing Dkk1 reveal no evidence of enhanced cell death (Purro and Salinas, unpublished data). Cell death is a protracted process; in contrast Dkk1 induces presynaptic disassembly and elimination within 10 minutes. I reason the discrepancy between my data and the above studies by arguing that it is the significant loss of synapses, induced by Dkk1, that promotes cell death rather than Dkk1 activity itself. A further discrepancy is that I used lower concentrations of Dkk1 than many of the studies cited here. The notion that Dkk1 aberrantly affects synapse stability is also consistent with current hypotheses for synapse dysfunction and elimination contributing to neuronal death.

In AD, synapse loss correlates with the accumulation of soluble Aβ prior to the onset of neuronal cell death (Hsia et al., 1999; Lacor et al., 2007; Shankar et al., 2007). However, mechanisms that relate soluble Aβ and synapse loss have been poorly understood. Research from our lab demonstrates that soluble Aβ induces synapse disassembly and loss in hippocampal brain slices within 3 hours (Purro, Dickins and Salinas, submitted). Acute exposure of Aβ induces transcription of Dkk1 mRNA; and critically, Dkk1 neutralizing antibodies block Aβ induced synapse loss. Together with my studies of Dkk1 induced synapse disassembly, these findings provide important insights into the mechanism by which Aβ induces synapse loss (Figure 7.3). Whilst further research is required to understand how Aß induces the expression of Dkk1 in neurons, these studies suggest the exciting potential of Dkk1 as a possible drug target for the treatment of AD.

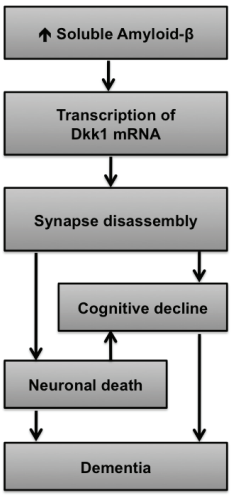


Figure 7.3: Model for Dkk1 in the progression of Alzheimer's disease. Increased levels of soluble Amyloid-β stimulate transcription of Dkk1 mRNA and loss of synapses in brain slices. Dkk1 induces rapid synapse disassembly by a process of synaptic protein dispersal leading to synapse elimination. Studies from our lab reveal elevated Dkk1 mRNA and synapse loss within 3-hours of soluble Amyloid-β exposure. It has been suggested that Dkk1 directly stimulates neuronal cell death. However, in our studies we do not detect evidence of cell death. We propose that Dkk1 induces synapse disassembly and elimination, which subsequently stimulates cell death. Loss of synapses correlates with cognitive decline. Our findings relate increased levels of soluble Amyloid-β with a mechanism for synapse loss. We propose that Dkk1 induced synapse elimination is an early event in the progression of Alzheimer's disease. The notion that synapse assembly precedes cell death is in accordance with current models for the progression of AD.

7.5. Conclusions

This thesis work demonstrates two key findings. First, Wnt7a stimulates presynaptic assembly of synaptic vesicles and the active zone, with coordinated clustering and apposition of postsynaptic proteins. The synaptogenic effect of Wnt7a is specific to the assembly of excitatory synapses, as inhibitory synapse formation is not promoted. A divergent canonical pathway, that bifurcates downstream of Gsk3β, regulates presynaptic assembly of excitatory synapses. Secondly, I demonstrate that Wnt signaling regulates the maintenance of synapses in young and mature neurons, which is a novel role for Wnt signaling in the CNS. These findings provide new insights into molecular mechanisms that link synapse assembly with synaptic maintenance and synapse disassembly.

7.6. Acknowledgments

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Without the support of my husband Roger, I would never have been able to embark upon my undergraduate or graduate studies. So, I want not only to acknowledge you, but also to dedicate this thesis to you.

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